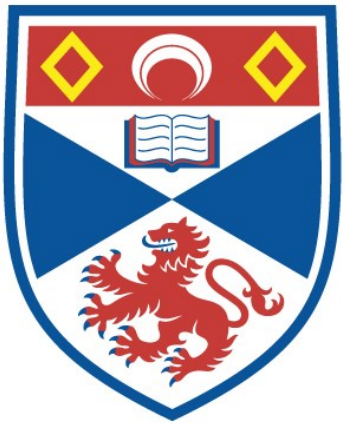
A NEW METHOD FOR CULTURING DOGFISH SHARK (SCYLIORHINUS CANICULA) RECTAL GLAND EPITHELIAL CELLS

David S. Nelson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1996

A new method for culturing dogfish shark (*Scyliorhinus canicula*) rectal gland epithelial cells

DAVID S. NELSON

ΑΙΕΝ ΑΡΙΣΤΕΥΕΙΝ

Declaration

a) I, David Spencer Nelson, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or qualification.

Signed Date March 24, 1995

b) I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance No. 12 in October 1993, and as a candidate for the degree of M.Phil in October 1993.

Signed Date March 24, 1995

c) We hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of M.Phil.

Signature of Supervisors Date March 27 1995

28/3/95

This thesis is dedicated to Mom, Dad, Mike, and Dan

Abstract

1. Dogfish, *Scyliorhinus canicula*, rectal gland epithelial cells were successfully cultured using two different techniques: 1) a perfusion based technique and 2) a modified Valentich's technique (Valentich, 1991).

2. Growth stages of cultures were monitored, showing attachment of tubules at approximately two days with a complete monolayer formed between seven and ten days. Cultures were able to be maintained for up to twenty days. Photos were taken illustrating epithelial cell migration and cell viability.

3. Administration of Ca^{+2} and Mg^{+2}-free Ringer + 2 mM ethylenediamine tetra-acetic acid (EDTA) + 1% trypsin successfully reduced cultures growing in 96-well plates to single cells after a time course of 20 min to allow for accurate cell counts of approximately 22,000 cells per well.

4. 10^{-6} M shark C-type natriuretic peptide (sCNP) induced stimulation of cGMP in cultured rectal gland epithelial cells

over a time course of 240 sec with maximal stimulation occurring at 180 sec. Limited experiments with scyliorhinin II and rectin showed little effects in stimulating cGMP.

Acknowledgements

I would like to express my thanks to Dr. Neil Hazon and Dr. Gordon Cramb for their support and encouragement throughout this project. I would also like to thank them for so graciously sharing their knowledge and experience with me and for welcoming me into their research groups and making me feel at home.

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Finally, my thanks go to the Lord, who can make all things possible and to Mom, Dad, Mike, and Dan for all their love, encouragement, and support. I also would like to thank Grandma Nelson, the Stitts and Jakalas, David, Todd, and Paul and their families for remembering me and keeping my letter box full, and to Grandpa Nelson and Grandma and Grandpa Spencer, who will never be forgotten.

Abbreviation

ADH	antidiuretic hormone
cAMP	cyclic adenosine 3', 5'-monophosphate
TMACl	tetramethylammonium
VIP	vasoactive intestinal peptide
dbcAMP	dibutyryl cAMP
TEA^+	tetraethylammonium
ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
CNP	c-type natriuretic peptide
VNP	ventricular natriuretic peptide
I_{sc}	short-circuit current
AP-III	atriopeptin III
AP-I	atriopeptin I
ANP II	Atrial natriuretic peptide II
cGMP	guanosine 3', 5'-monophosphate
NPY	neuropeptide Y
8-CPT-cAMP	8-chlorophenylthio-cAMP
TMAO	trimethylamine N-oxide
EDTA	ethylenediamine tetra-acetic acid

EGTA	ethyleneglycol-bis-(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid
SNP	sodium nitroprusside
EBAO	ethidium bromide acridine orange
sCNP	shark c-type natriuretic peptide
IBMX	3-isobutyl-1-methyl-xanthine
PCA	perchloric acid
UII	urotensin II

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1.0 Introduction

1.1 Models of epithelial transport

Epithelial sodium transport is employed by many organisms of different environments and habitats as a method for controlling salt and water balance. This balance is a crucial requirement for the body to maintain an internal fluid homeostasis. Salt, or more directly the sodium ion, plays an important part in epithelial transport at the cellular level by moving to and from the apical and basolateral surfaces to establish electrochemical gradients across membranes. These electrochemical gradients can be used to induce the active transport of other substances and ions such as Cl^-, HCO_3^-, glucose, and K^+. Not only are electrochemical gradients produced but also osmotic gradients, to induce the absorption or secretion of water across the epithelial boundaries in order to satisfy a particular environmental demand. These environmental demands may present themselves in different forms, such as the ability of a fish to cope with a high or low salinity environment; the ability of a mammal to live in a hot, desert-like environment where little or no water is present in contrast to an environment in which water is plentiful; or even the ability of a sea bird to cope with life in high salinity saltwater. All of these situations impart an urgency upon the organism to control the amount of water that is leaving its body. This ability depends on epithelial sodium transport to establish gradients for the movement of water.

As often happens in nature, organisms develop different yet similar methods of maintaining water balance. These similarities allow for comparison between organisms and provide very useful models for studying epithelial sodium transport. Epithelial transport in the thick ascending limb of the nephron of the mammalian kidney, the avian salt glands, the chloride cell in teleosts, and the elasmobranch rectal gland are all very similar in the sense that each one possesses a mechanism of epithelial sodium transport. In each case, they contribute to the overall good of maintaining homeostasis of ions and water balance.

1.2 Mammalian kidney

The mammalian kidney consists of approximately 2 million nephrons which act as filtration units for blood plasma to remove waste products while retaining important ions and substances essental for normal physiology. Filtration begins at the glomerulus, a tight ball of capillaries which exhibit selective filtration properties. Reabsorpton of essential substances filtered occurs in different regions of the nephron with the proximal tubule being quantitatively of most importance. Proximal tubule reabsorption is an obligatory process with between 70-90% of reasorption of essential substances occurring in this region by a variety of both active and passive transport pathways.

Following the late proximal tubule and passing into the inner

medula, the loops of Henle, composed of the thin descending and thin and thick ascending limbs, have varying reabsorptive responsibilities depending upon which part of the loop is considered. Each limb has different permeability characteristics, allowing for selective reabsorption of solutes or water (Figure 1.1). The thin descending limb is impermeable to sodium and chloride but allows water to pass through. This concentrates the solutes within the filtrate. On the other hand, the thin and thick ascending limbs are impermeable to water and permeable to sodium, chloride, and urea.

A portion of the thick ascending limb lies in the outer medulla and a portion in the cortex. The medullary portion functions to produce a maximal hypertonic environment within the medulla against small concentration gradients by actively transporting sodium into the interstitium. Similarly, the cortical portion functions to dilute the tubule fluid against high concentration gradients by actively transporting sodium into the interstitium via Na-K-ATPase. The thick ascending limb is characterized by a Na-K-2Cl cotransporter located in the apical membrane and Na-K-ATPase located in the basolateral membrane (Figure 1.2). The active transport of sodium out of the cell into the interstitial fluid by way of the Na-K-ATPase creates a concentration gradient for sodium entry into the cell via the Na-K-2Cl cotransporter. A potassium channel located in the apical membrane also acts to decrease

Figure 1.1

Figure 1.1 Movements of ions and solutes in the mammalian nephron.

(from Carola et al., 1990).

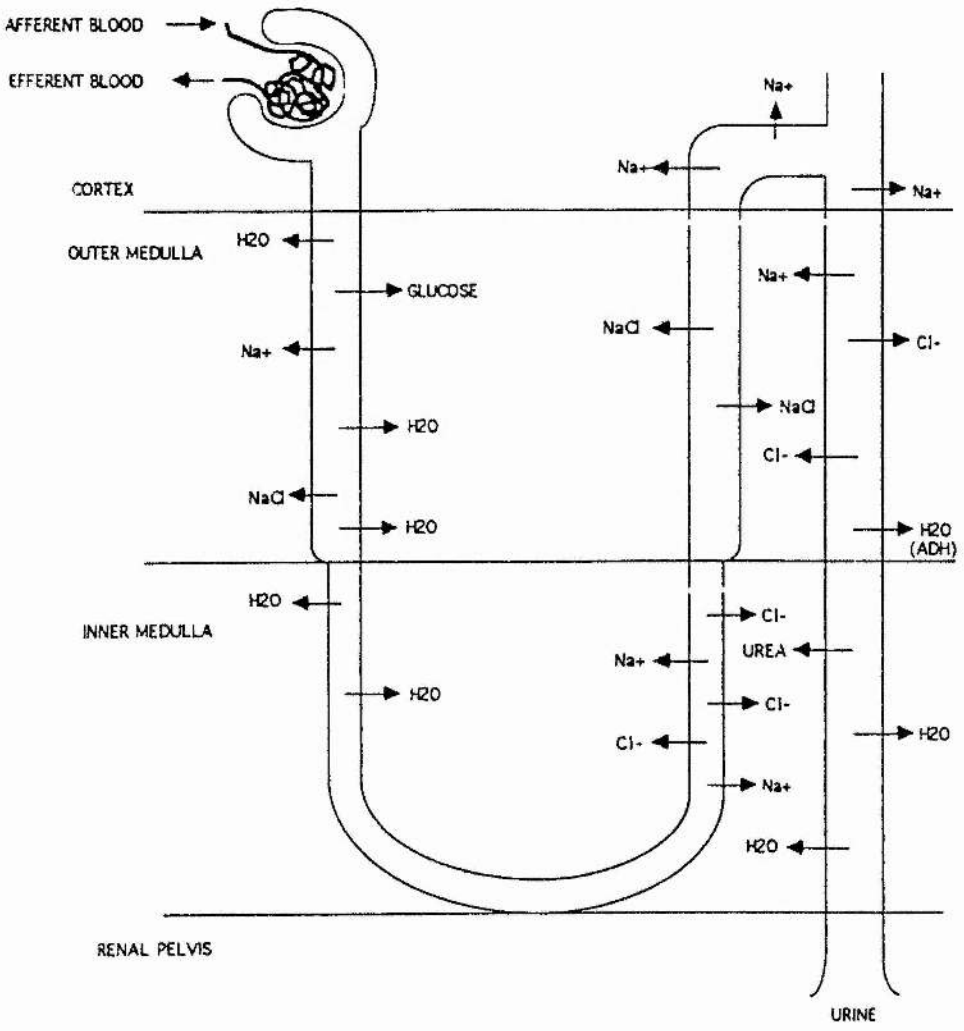
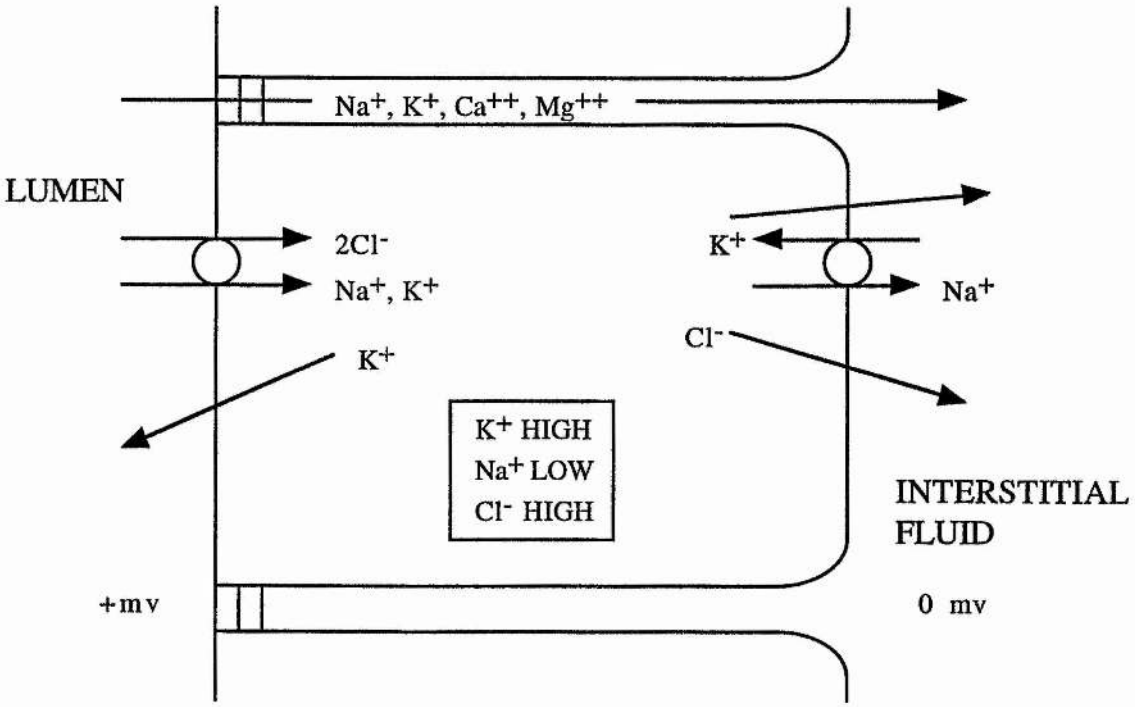

AFFERENT BLOOD
EFFERENT BLOOD
CORTEX
OUTER MEDULLA
INNER MEDULLA
RENAL PELVIS
URINE
H2O
GLUCOSE
Na+
NaCl
H2O
NaCl
H2O
H2O
H2O
Na+
NaCl
Cl-
NaCl
Cl-
H2O
(ADH)
Cl-
UREA
Cl-
H2O
Na+
H2O
Na+
Na+
Na+
Cl-
Cl-
Na+
H2O

Figure 1.2

Figure 1.2 Mechanism for epithelial sodium transport in the thick ascending limb of Henle in the mammalian kidney.

(from Brenner er al., 1987).


LUMEN
Na+, K+, Ca++, Mg++
2Cl-
Na+, K+
K+
K+
Na+
Cl-
K+ HIGH
Na+ LOW
Cl- HIGH
INTERSTITIAL FLUID
+mv
0 mv

potassium concentration gradient by allowing potassium to diffuse back into the lumen. Chloride moves into the cell drawn by the sodium concentration gradient, leaving the lumen positively charged. This lumen positive charge functions to drive sodium, potassium, calcium, and magnesium through paracellular pathways into the interstitial fluid. Now a very dilute fluid is left in the lumen (Brenner et al., 1987).

The distal convoluted tubule follows the thick ascending limb as it re-enters the cortex of the kidney. Acting much the same as the proximal tubule, the distal convoluted tubule passively reabsorbs sodium across the luminal membrane because of the electrochemical potential gradient and then actively passes the sodium across the basolateral membrane by way of Na-K-ATPase. Chloride usually can follow passively. Water reabsorption depends in large part on the presence of antidiuretic hormone (ADH) which causes the distal convoluted tubule to become more permeable to water. In the presence of ADH, water reabsorption will occur.

Finally, the end of the filtrate journey terminates after the collecting ducts. The collecting ducts originate in the cortex, pass through the outer and inner medulla, and end in the renal pelvis, where they empty into the ureter. Although only approximately 1.5% of sodium is absorbed here, reabsorption occurs in much the same way as in the distal convoluted tubule and proximal tubule (Figure 1.1).

Sodium passively crosses the apical membrane because of an electrochemical gradient and is actively transported into the peritubular fluid, using Na-K-ATPase located in the basolateral membrane. Again, reabsorption of chloride is accomplished mainly by passive diffusion.

Water reabsorption, as seen in the distal convoluted tubule, again depends on the presence of ADH. All water reabsorption in the collecting duct occurs passively due to a process known as countercurrent multiplication (Brenner et al., 1987).

In the absence of ADH, little water will be absorbed, and a highly dilute urine will be excreted. In the presence of ADH, the walls of the distal convoluted tubule and collecting duct will become permeable to water, and water reabsorption occurs down the osmotic gradient created by countercurrent multiplication. In addition to increasing water permeability, ADH also increases intermedullary collecting duct permeability to urea, thereby enhancing the concentration gradient for passive reabsorption of water. Thus, much of the water in the collecting duct is reabsorbed, and the resulting urine is highly concentrated.

1.3 Avian salt gland

The preceding paragraphs included a very basic discussion of how the mammalian kidney is able to regulate the amount of water that is reabsorbed through its use of epithelial sodium transport. The

mammalian kidney is not the only organ that makes use of epithelial sodium transport to control water reabsorption; the avian salt gland regulates salt and water balance by essentially the same mechanisms.

The salt glands are located on the top of the skull just above the orbit of each eye. These paired salt glands, connected through a duct to the nasal cavity, are primarily found in marine birds because they inhabit a high salinity environment, but they are also found in terrestrial birds (Schmidt-Nielsen, 1979). In contrast to the mammalian kidneys which are constantly active, the avian salt glands function only when the bird has undergone a high salt intake either by food or water.

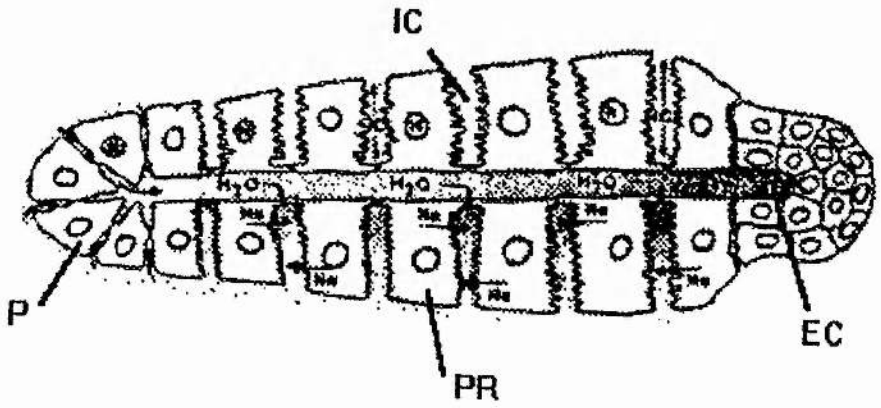
The salt gland contains lobules filled with secretory tubules which surround a central excretory canal (Figure 1.3). At the terminal end of the secretory tubule away from the central excretory canal are located the peripheral cells which are small in size and function to secrete an isotonic solution into the lumen of the secretory tubule (Ellis et al., 1977). As the solution passes down the lumen, water is selectively reabsorbed down osmotic gradients through larger cells known as principal cells. The apical surfaces of the principal cells bind together to create the luminal canal.

The osmotic gradients are set up by the principal cells in the following manner. NaCl is supplied by the blood and passively diffuses into the principal cells. Na-K-ATPase located on the lateral plasma

Figure 1.3

Figure 1.3 Avian salt gland lobule.

The avian salt gland is a specialized organ for excreting a concentrated salt solution by reabsorbing water from the lumen of the lobule. The gland is composed of peripheral cells (P), principal cells (PR), an excretory duct (EC), and intercellular channels (IC) (from Ellis et al., 1977).

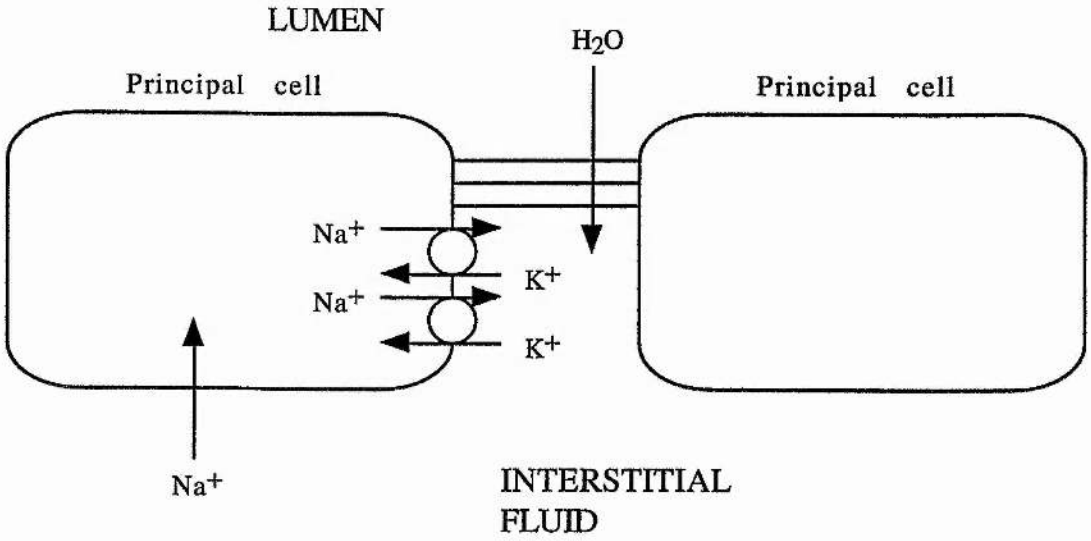

IC
P
PR
EC

membranes (Ernst, 1972) actively transport sodium into the lateral spaces or intercellular channels (Figure 1.4). This active transport of sodium out of the cell creates the gradient for passive diffusion of sodium into the cell. Once the sodium has been transported into the intercellular channels, an osmotic gradient is set up for the passive diffusion of water through the permeable cell junctions binding the principal cells together. The water cannot pass through the apical surfaces of the principal cells because that surface is impermeable to water similar to the thin and thick ascending limbs of Henle in the mammalian kidney. To increase the gradient, the highest concentrations of sodium are found near the luminal surface (Ellis et al., 1977). The fluid left in the lumen is now hypertonic because water has been reabsorbed and can be excreted by way of the central excretory canal (Ellis et al., 1977).

Although the avian method of reabsorbing water through the use of epithelial sodium transport is a little different from the method found in the mammalian kidney, there are similarities such as the use of Na-K-ATPase, the production of a hypertonic salt excretion if necessary, the use of osmotic gradients to drive water to its desired locations, and areas of epithelium which are impermeable to water. The differences arise in the purpose of sodium transport. The primary goal of the nephron is water and ion reabsorption, as opposed to the primary goal

Figure 1.4

Figure 1.4 Mechanism involved in the creation of a concentrated salt excretion in the avian nasal gland.


LUMEN
H_2O
Principal cell
Principal cell
Na^+
K^+
Na^+
K^+
Na^+
INTERSTITIAL FLUID

of the salt gland to excrete a concentrated salt solution.

1.4 Teleost chloride cell

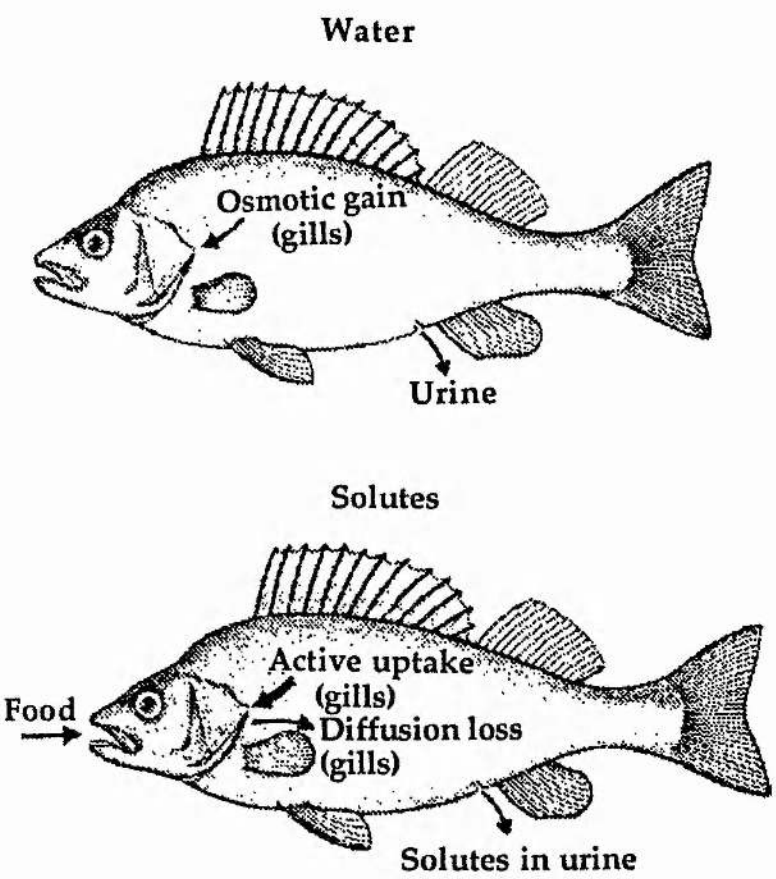
The teleost chloride cell is another model that can be used to study epithelial sodium transport. Because fish encounter environments which can be vastly different with respect to salinity, they must have some mechanism to control the amount of water that is entering and leaving their system. Teleost fish accomplish this task through the use of chloride cells. Chloride cells, like those cells found in the mammalian kidneys and in the avian salt glands, are specially suited to control the salt and water balance within the fish.

The teleost fish gill has many responsibilities, including gas exchange, regulation of ion flow, excretion of nitrogenous waste, and the maintenance of an acid-base balance (Perry and Walsh, 1989). As alluded to earlier, euryhaline teleost fish often inhabit both freshwater and saltwater environments and must have a method to control salt and water fluxes across the gill epithelium. A fish in freshwater typically wants to remain hypertonic with respect to its environment and thus must have a method to remove excess water which enters its body down an osmotic gradient (Figure 1.5). A fish in saltwater has just the opposite problem: it wants to remain hypotonic with respect to its environment and thus must have a method for retaining water which

Figure 1.5

Figure 1.5 Control of ions and solutes by the freshwater teleost. (from Schmidt-Nielsen, 1979).

FRESH-WATER TELEOSTS



would otherwise pass out of its body driven by an osmotic gradient (Figure 1.6). The mechanism through which this balance is obtained is the chloride cell located in the teleost fish gill, a site of passive transfer of water (Maetz, 1971).

The teleost gill is fed by the afferent blood vessel and drained by the efferent blood vessel (Figure 1.7). The bottom portion of the gill is supported by a row of cartilage to give the gill support. Branching from the afferent vessel are four branchial arches, each possessing two rows of filaments extending down each side of each arch. It is on the outer boundaries of these filaments where the chloride cells are found (Maetz, 1971; Keys and Willmer, 1932).

Chloride cells are not the only cell type to inhabit the stratified epithelium of the teleost gill; but there are also populations of pavement cells, mucous cells, and non-differentiated cells (Degnan et al., 1977). For the most part, the pavement cells occupy a large part of the epithelial surface, except where the chloride cells and mucous cells come in contact with the external environment. The non-differentiated cells extend down the sides of the chloride cells and fill up the space between the basal lamina and the pavement and mucous cells on the surface, except where the chloride cells interrupt. The chloride cells make up approximately 50-70% (Karnaky and Kinter, 1977) of the epithelium of the opercular skin in killifish (*Fundulus heteroclitus*) and seem to be

Figure 1.6

Figure 1.6 Control of ions and solutes by the marine teleost. (from Schmidt-Nielsen, 1979).

MARINE TELEOSTS

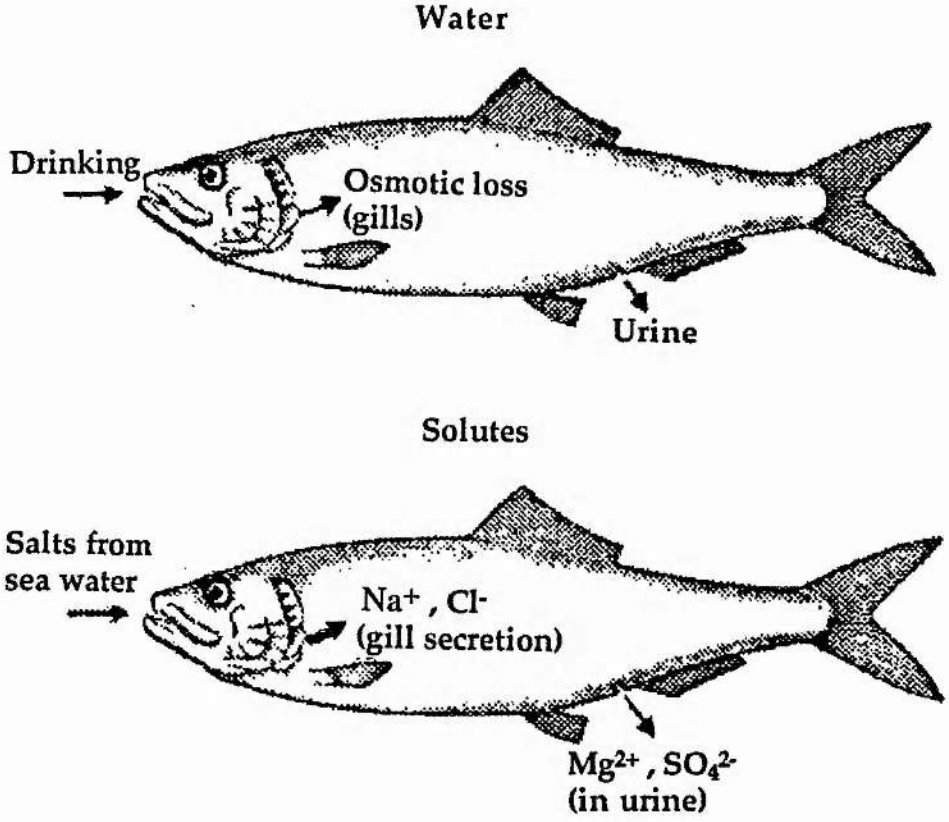
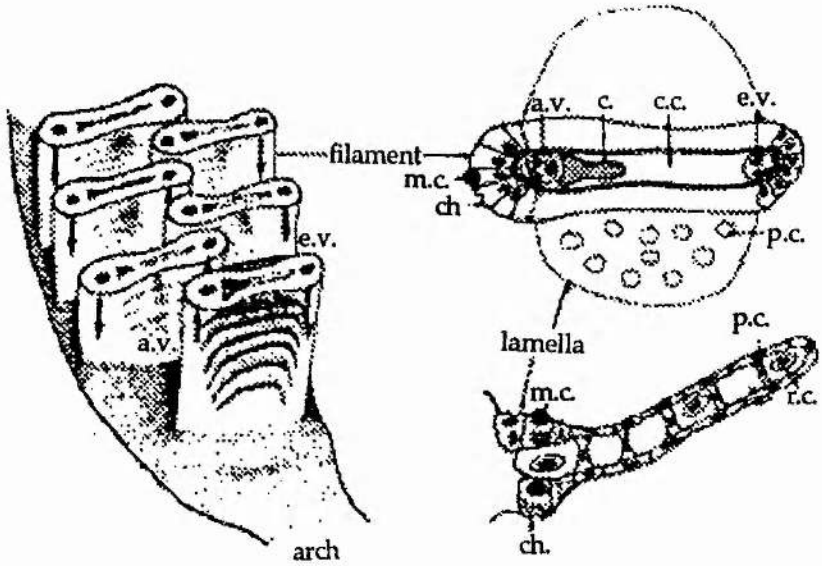


Figure 1.7

Figure 1.7 Morphology of the teleost gill.

Three pairs of gill arches can be seen accompanied by two rows of filaments with lamellae extending from each side of the filaments. a.v. and e.v., afferent and efferent vessels with red cells (r.c.); c., cartilagenous support; c.c., central vascular compartment; m.c., mucous cell; ch., chloride cell; p.c., pillar cells (from Maetz, 1971).


filament
m.c.
ch
e.v.
a.v.
arch
a.v.
c.
c.c.
e.v.
p.c.
lamella
p.c.
m.c.
r.c.
ch.

more abundant in fish adapted to saltwater (Keys and Willmer, 1932).

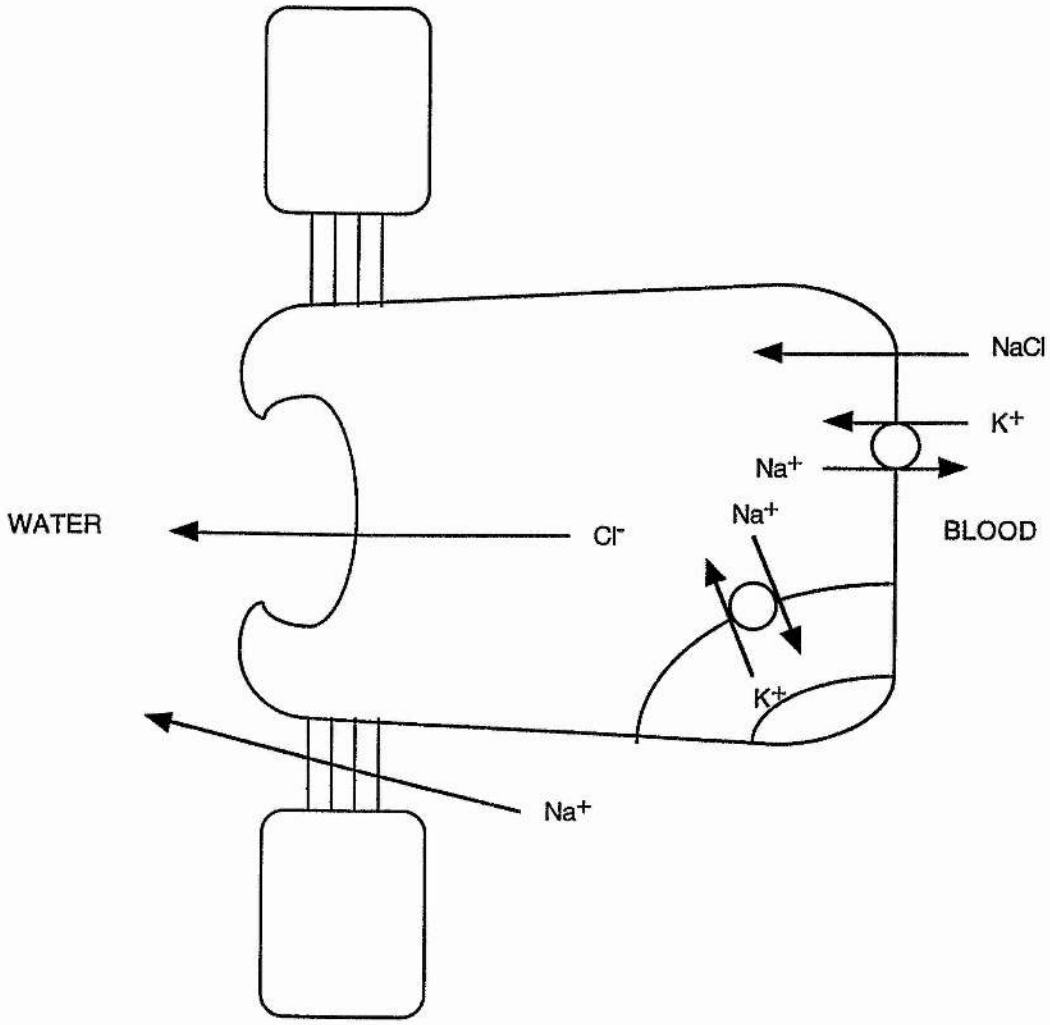
Morphologically, the chloride cell has a characteristic apical crypt which comes in contact with the external environment (Figure 1.8). These apical crypts seem to develop after adaptation to seawater as they are not present in killifish adapted to tapwater (Copeland, 1950; Philpott and Copeland, 1963). Just below the apical crypt is a thin layer of cytoplasm which contains microtubules, microfilaments, polyribosomes, and, in particular, various small vesicles. Some of these vesicles appear to be fused with the apical crypt membrane (Hootman and Philpott, 1979). This indicates that the apical crypt may have an important role in chloride secretion which must occur in seawater (Degnan et al., 1977) and will be discussed later.

The chloride cell contains a high number of mitochondria and a large, pronounced nucleus. Passing through the cell is a highly branched tubular system (Degnan et al., 1977). Not only is the apical crypt more evident in seawater adapted fish, but the tubular system is also more pronounced (Karnaky et al., 1976a). The basolateral membrane is characterized by an extensive amount of infolding (Kirschner, 1991) possibly for an increased amount of surface area in contact with the basal lamina.

The chloride cell, being the primary source of osmoregulation in teleosts (Keys and Willmer, 1932), must have a method for pumping

Figure 1.8

Figure 1.8 Chloride secretion and epithelial sodium transport in the chloride cell of the teleost fish.


NaCl
K+
Na+
WATER
Cl-
Na+
BLOOD
K+
Na+

ions in and out of the cell. Similar to the thick ascending limb cells of the mammalian kidney and the avian salt gland, the teleost makes use of Na-K-ATPase and the basolateral NaCl cotransporter (Foskett et al., 1983) (Figure 1.8). According to Karnaky et al. (1976b), nearly all Na-K-ATPase found in the gill is associated with chloride cells. The Na-K-ATPase appears to be located in the basolateral cell surface which puts it in close contact with the interstitium. Because the Na-K-ATPase is located in the basolateral membrane, it can be assumed that sodium is being actively transported from inside the chloride cell into the interstitial fluid (Karnaky et al., 1976b). This makes sense for fish adapted to fresh water because they want to absorb sodium in order to remain hyperosmotic with respect to their environment, but it leaves some question as to how a fish adapted to seawater excretes sodium to remain hypoosmotic with respect to its environment. Karnaky et al. (1976b) found no evidence of the presence of Na-K-ATPase in the apical crypt.

Hootman and Philpott (1979) suggest that the Na-K-ATPase pumps sodium from the interior of the cell into the interstitial fluid, creating a gradient for passive diffusion of sodium back into the cell. As sodium diffuses into the cell, chloride may be brought along as part of a cotransport mechanism. The chloride would then be excreted from the cell down an electrical gradient. Foskett et al. (1983) suggest that

sodium then exits the cell driven by a serosa-positive voltage and moves into the external environment via permeable cell junctions between the chloride cells and adjacent non-differentiated cells or accessory cells. This is similar to the permeable junctions found in the avian salt glands. Degnan et al. (1977) determined that both sodium and chloride were required for a net transepithelial potential difference and that net chloride fluxes across the epithelia from the basolateral to apical surfaces and into the external media were present in both freshwater and saltwater-adapted *Fundulus heteroclitus*.

In contrast to Karnaky et al. (1976b), Hootman and Philpott (1979) did find some evidence of Na-K-ATPase localization near the apical membrane in the vesicular system of the pinfish. This indicates that the vesicles may play some part in the osmoregulation of the cell.

Not only is epithelial sodium transport present in the gills of teleost fish, but it also increases with increasing salinities. Karnaky et al. (1976a) found that Na-K-ATPase activity in the pupfish *Cyprinodon variegatus* increases through adaptation to rising salinities. Adaptation to varying salinities was compared in 50% seawater, 100% seawater, and 200% seawater. Na-K-ATPase activity in 100% seawater was 1.6 times greater than that found in 50% seawater. Activity in 200% seawater was 6.3 times greater than that found in 50% seawater. This indicates that the chloride cell is removing excess chloride from the fish in order

to maintain its hypotonicity with respect to its environment. Hootman and Philpott (1979) similarly found that Na-K-ATPase activity increases with transfer of the fish from freshwater to artificial seawater. These data indicate that the chloride cell has the ability to turn itself on and off just as the avian salt gland can have varying periods of activity depending on the environment it encounters.

As each of the aforementioned models provides examples of epithelial sodium transport, they are all characterised by possessing a large population of cells which are not involved in sodium transport, thus making it rather difficult to study effectively the mechanism at the cellular level. In contrast, the elasmobranch rectal gland is almost a homogeneous collection of chloride-secretory epithelial cells (Valentich, 1991). The shark rectal gland consists of tightly packed secretory tubules with epithelial cells lining these tubules (Valentich and Forrest, 1991). The presence of connective tissue can be found, but only in small areas. Because the rectal gland is composed almost entirely of chloride-secretory cells, it lends itself as an excellent model for the study of epithial sodium transport via <u>in vitro</u> perfusion or, in particular, via cell culture. In contrast, the other transport models, such as the teleost chloride cell, may be difficult to study in culture due to the heterogeneity of cell types.

1.5 Rectal gland

1.5.1 General information

Scyliorhinus canicula occupy a habitat off the west coast of Britain. They typically inhabit marine environments and grow to a size of approximately 0.75 m and have a mass of approximately 1.0-1.5 kg in adulthood. The elasmobranchs, in contrast to the teleosts, typically maintain their body fluids as isosmotic or slightly hyperosmotic to the seawater, but they still undergo a sodium load from seawater and food (Figure 1.9). The kidney must then be able to regulate ion concentration in order to achieve this relationship (Henderson et al., 1988).

The kidney of *Scyliorhinus canicula* contains nephrons spread throughout mesial tissue and lateral bundles. The nephron makes two main turns in the mesial tissue and two in the lateral bundle (Figure 1.10). A low urea concentration is found within the bundle surrounding the end of the renal tubules. As a result, urea is passively reabsorbed from the filtrate into the blood at this part of the renal tubule (Hentschel, 1987). High urea content in the blood allows *Scyliorhinus* to remain isosmotic or slightly hyperosmotic to its environment.

The rectal gland in *Scyliorhinus canicula* is located approximately 5 cm anterior to the anus and lies at the posterior end of the intestine

Figure 1.9

Figure 1.9 Ion and solute concentrations within the shark.

The shark maintains an internal environment which is slightly hypertonic with respect to its external environment.

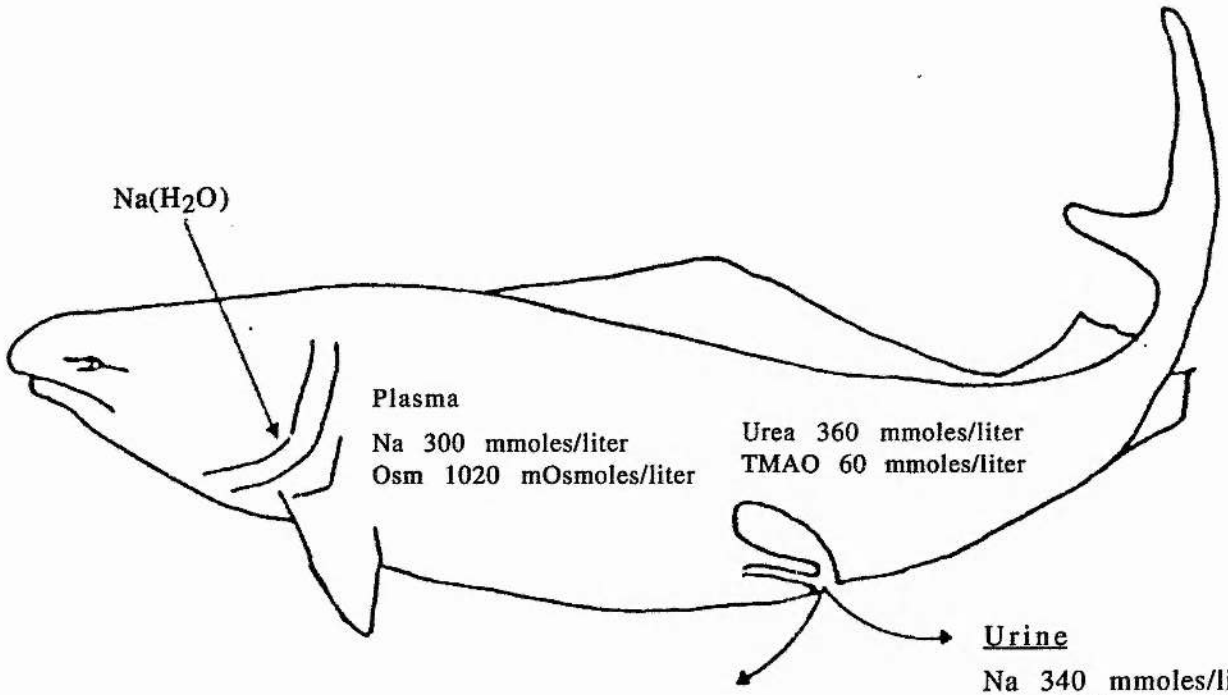
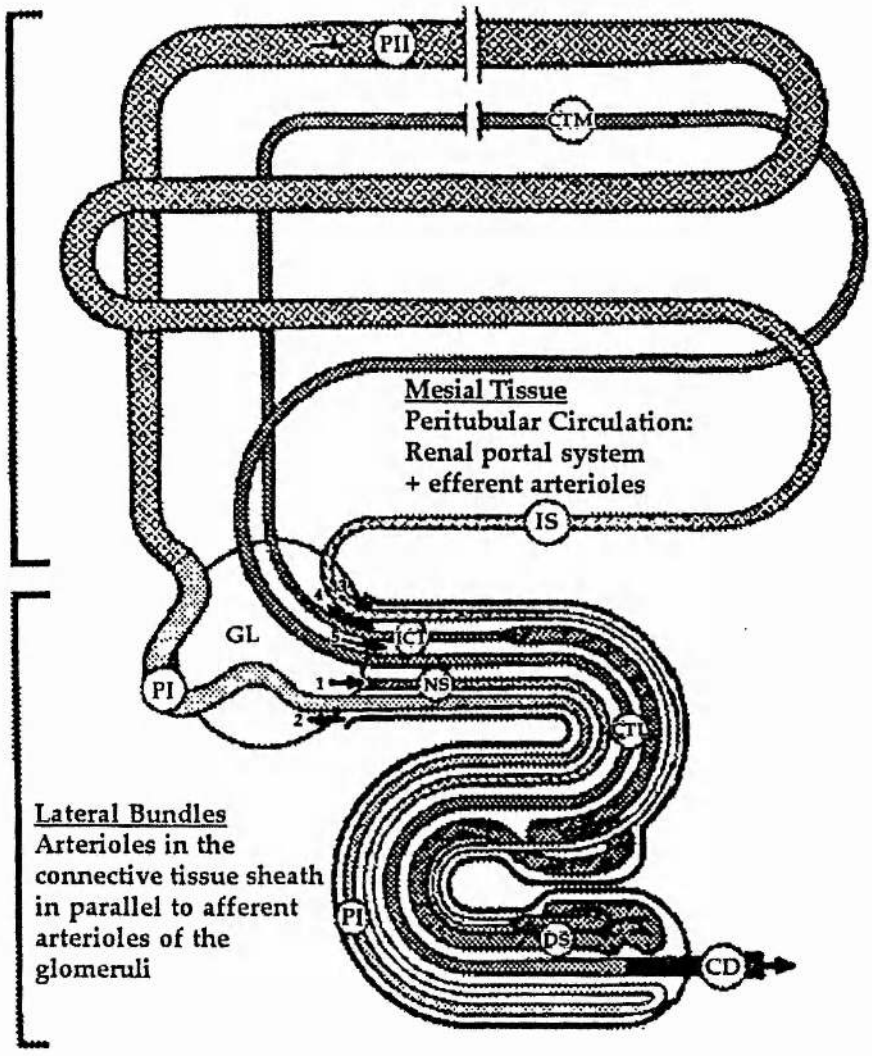

Na(H_2O)
Plasma
Na 300 mmoles/liter
Osm 1020 mOsmoles/liter
Urea 360 mmoles/liter
TMAO 60 mmoles/liter
Urine
Na 340 mmoles/liter
Osm 800 mOsmoles/liter
SW
Na 470 mmoles/liter
Osm 1000 mOsmoles/liter
Rectal gland secretion
NaCl 1080 mmoles/liter
Osm 1020 mOsmoles/liter

Figure 1.10

Figure 1.10 Nephron of the dogfish shark kidney (*Scyliorhinus canicula*).

The nephron is located in both the mesial tissue and the lateral bundles of the kidney. GL glomerlus; NS neck segment; PI proximal tubule segment part I; PII proximal tubule segment part II; IS intermediate segment; DS distal segment; ICT initial collecting tubule; CTM mesial collecting tubule; CTL lateral collecting tubule; CD collecting duct (from Hentschel, 1987).


PII
CTM
Mesial Tissue
Peritubular Circulation:
Renal portal system
+ efferent arterioles
IS
GL
PI
1
2
NS
CT
CT
PI
DS
CD
Lateral Bundles
Arterioles in the
connective tissue sheath
in parallel to afferent
arterioles of the
glomeruli

(Nilsson and Holmgren, 1988). Fed by the rectal gland artery and drained by the rectal gland vein and duct, the rectal gland is cylindrical in shape approximately 1.0 cm in length with a central canal or duct passing through the middle. The dense population of tubules composing the rectal gland enter into this central duct (Figure 1.11) and are separated by very thin layers of connective tissue (Valentich and Forrest, 1991). In addition to the connective tissue, small amounts of mesothelial and vascular cell types are also present along with fibroblasts from the interstitial matrix and epithelial cells from the central duct. It should be noted again that the chloride-secretory epithelial cells are the predominant cell type within the gland.

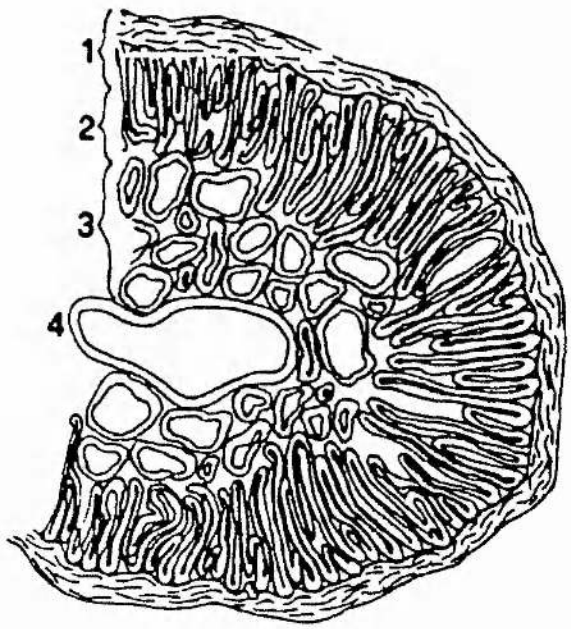
The elasmobranch rectal gland is important to the maintenance of homeostasis within the dogfish. Because elasmobranchs live in an environment with such high salinities, the dogfish must be able to secrete large quantities of salt while remaining isotonic or slightly hypertonic with respect to the environment. Solomon et al. (1984a) found blood plasma in *Squalus acanthias* to be 1000 ± 25 mosmol/kg and seawater to be 972 ± 16 mosmol/kg illustrating the slight hypertonicity of the plasma with respect to seawater.

The rectal gland also plays a role in regulation of volume expansion. According to Solomon et al. (1984a), the rectal gland is stimulated by volume expansion. Because the blood plasma is slightly

Figure 1.11

Figure 1.11 Diagrammatic cross-section of the dogfish rectal gland.

The gland can be divided up into four sections: 1) capsular and subcapsular zones, 2) layer of radial tubules, 3) inner zone of branching tubules, and 4) the central canal (from Masini et al., 1994).


1
2
3
4

hypertonic with respect to the seawater, water most likely diffuses into the fish across the gills, down an osmotic gradient, and raises fluid volume in the fish. This volume expansion may induce a stimulation in rectal gland activity which results in chloride secretion. The increase in osmolality of the lumen results in an increase in water secretion activity, effectively removing excess salt and water from the body (Solomon et al., 1984a). It is important to note that the rectal gland is in fact stimulated by volume expansion and not by an increase in solute concentration (Solomon et al., 1985).

1.5.2 Morphology

Individual dogfish rectal gland epithelial cells are characterized by a high density of mitochondria throughout the cell and lateral cell borders which are highly interdigitated with adjacent cell borders. Junctional complexes link the adjacent cells together. Tubulo-vesicles also occupy the cytoplasm near the apical plasma membrane (Valentich, 1991).

The dogfish shark rectal gland has various mechanisms for regulating salt excretion. Like the previously mentioned models, the epithelial cells also possess Na-K-ATPase and a cotransport mechanism for transport of ions. In addition, epithelial cells possess apical chloride channels and basolateral potassium channels for transporting chloride

and potassium across the apical and basolateral membranes, respectively. These apical chloride channels are "induced" by cyclic adenosine 3', 5'-monophosphate (cAMP) (Greger et al., 1985).

1.5.3 Chloride ion

Gögelein et al. (1987) determined that chloride is the ion responsible for the conduction as opposed to sodium by testing conduction in patch-clamped, isolated rectal gland tubule segments bathed in NaCl first, then KCl, and finally tetramethylammonium chloride (TMACl) solution. A conductance of 8pS was found when the segments were bathed in NaCl or KCl, but only negative voltages were found with TMACl in the bath. When the single channel current is plotted as a function of the clamp voltage, almost identical lines are seen for NaCl and TMACl. Thus the channel is impermeable to sodium and chloride is the only conducting ion.

1.5.4 Na-K-2Cl Cotransport

In order for chloride to be the conducting ion, it must find some way to enter the cell. Greger and Schlatter (1984a) hypothesized that there must be a mechanism for chloride entry into the cell due to high chloride activity within the cell. Upon addition of a potent Na-K-2Cl cotransport inhibitor, chloride activity within the cell dropped indicating

that the Na-K-2Cl cotransport is a major route for chloride entry into the cell. The Na-K-2Cl cotransporter actively transports chloride into the cell against an electrical gradient and a high intracellular chloride concentration (Silva et al., 1977). To indicate the importance of the Na-K-2Cl cotransporter, the rectal gland of the dogfish shark contains 10 to 100 times more Na-K-2Cl cotransport protein than other tissue in the body, making up approximately 2% of the total membrane protein (Forbush et al., 1992).

Greger and Schlatter (1984a) also noticed that secondary hyperpolarization and a decrease in transepithelial potential difference result when potassium is held in low concentrations in the bath perfusate. This indicates that potassium may play a role in the cotransport system. In fact, sodium and chloride do not enter across the basolateral membrane without the presence of potassium. Hannafin et al. (1983) noticed a 50% decrease in sodium uptake as potassium was reduced from 50 mM to 20 mM and sodium uptake is markedly reduced in the complete absence of potassium.

In a follow up study, Greger and Schlatter (1984b) and also Silva et al. (1977) found that chloride concentration within the cell dropped in response to a substitution of choline^{+} for sodium. Hannifin et al. (1983) found that sodium uptake is also dependent on chloride from data showing sodium uptake to be five times greater when exposed to

KCl as opposed to KNO_3. Silva et al. (1977) found that the movement of sodium into the cell is down an electrochemical gradient. Sodium therefore fills the third part of the cotransport system. The question still remained as to the ratio of entry for each of the three ions. When furosemide was added to the perfusate, the chloride concentration within the cell dropped almost twice as fast as the sodium concentration, indicating a $2Cl^-:Na^+:K^+$ relationship (Greger and Schlatter, 1984b). Furosemide is a potent inhibitor of Na-K-2Cl cotransport protein (Palfrey et al., 1984).

Once the cell has been stimulated, numerous changes within the cell occur. According to Greger et al. (1984c), changes in potential difference, resistance, and current are seen almost immediately after stimulation by a combination of 10^{-4} M dibutyryl-cyclic adenosine monophosphate (dbcAMP), 10^{-4} M adenosine, and 10^{-6} M forskolin (combined to ensure a fast response). Addition of these chloride secretory stimulants results in increased lumen negative transepithelial potential difference and a hyperpolarized basolateral potential difference. The transepithelial resistance falls as does the apical membrane resistance. A fall in these resistances indicates passage of ions across the cell membranes. Chloride activity from cell to lumen in the nonstimulated state is 59 μAcm^{-2} and in the stimulated state is

$267\mu Acm^{-2}$. Stimulation produces almost an 8-fold increase in current.

As might be expected, stimulation results in a drop in concentration of chloride within the cell. In contrast, the concentration of sodium within the cell increases upon stimulation accompanied by an increase in electrochemical potential difference for sodium. The potassium concentrations remain fairly stable (Greger et al., 1984c). The increase in sodium concentration is probably due to an increase in Na-K-2Cl cotransport activity.

In order for chloride secretion to be achieved at all, one of these steps must occur first following a particular stimulus. Greger et al. (1984c) conclude that the opening of the apical chloride channels may be the first step in the process supported by their data that the luminal conductance to chloride can be turned on in the presence of furosemide while the Na-K-2Cl cotransporter is inhibited. This indicates that the Na-K-2Cl cotransporter is not the primary event in stimulation.

The chloride concentration within the cell may be the controlling factor in overall rectal gland stimulation due to the fact that low intracellular chloride concentrations increase [^{3}H] benzmetanide binding site density (Lytle et al., 1992). The level of benzmetanide binding indicates the activity level of the Na-K-2Cl cotransport system and is a potent inhibitor of Na-K-2Cl cotransport (Forbush III et al., 1992a). Thus the Na-K-2Cl cotransporter is trying to move more chloride into

the cell in response to low chloride levels.

Greger et al. (1984c) and Forbush III et al. (1992a) also suggested that intracellular chloride activity may play a role in activating Na-K-2Cl cotransport activity. It is suggested that the number of Na-K-2Cl cotransporters increase upon stimulation based on data that [^{3}H] benzmetanide binding increases by sixteen times in vasoactive intestinal peptide (VIP) stimulated rectal glands (Forbush III et al., 1992a). Cell shrinkage induced by placing tubules in a hypertonic binding medium also was able to stimulate Na-K-2Cl cotransport activity as seen through [^{3}H] benzmetanide binding (Lytle and Forbush III, 1992b).

1.5.5 Na-K-ATPase

Once the Na-K-2Cl cotransporter transports these ions into the cell, the cell must have a method for removing them again to avoid cell swelling and to maintain an electrochemical gradient. Located in the basolateral membrane along with the Na-K-2Cl cotransporter is the enzyme Na-K-ATPase (Karnaky et al., 1976). Bonting (1966) reported that Na-K-ATPase occurs at high levels in the elasmobranch rectal gland and that it is inhibited by sodium- or potassium-free media or the presence of ouabain. Ouabain produces complete inhibition at 10^{-4} M

(Bonting, 1966; Silva et al., 1977). This indicates that both sodium and potassium are necessary for the functioning of Na-K-ATPase just as they are for the Na-K-2Cl cotransporter. When the Na-K-ATPase is inhibited, chloride secretion is inhibited linking the two processes (Silva et al., 1977).

The function of the Na-K-ATPase is to maintain low levels of sodium within the cell, producing an electrochemical gradient for passive movement of sodium into the cell by way of the Na-K-2Cl cotransporter. Addition of 10^{-4} M ouabain produces the expected rise in intracellular sodium due to the inability of Na-K-ATPase to transport sodium out of the cell (Shuttleworth and Thompson, 1980). Interestingly, there is no accumulation of sodium upon 10^{-4} M ouabain addition in the presence of 5×10^{-4} M furosemide because now sodium entry into the cell via the Na-K-2Cl cotransport has been inhibited. Sodium, then, is actively transported from the intracellular space into the blood and potassium is actively transported from the blood into the cell via Na-K-ATPase (Silva et al., 1977; Silva and Myers, 1986; Greger and Schlatter, 1984a; Greger et al., 1984c).

Increased cellular sodium concentration appears to be responsible for Na-K-ATPase stimulation from data showing low cellular sodium concentrations in nonstimulated cells (Greger et al., 1984c); as a result,

Na-K-ATPase maintains its role of keeping levels of cellular sodium low to produce a gradient for the sodium entry via the Na-K-2Cl cotransport. Therefore, the more sodium moved into the cell by the Na-K-2Cl cotransport, the more active the Na-K-ATPase will be, indicating that Na-K-2Cl cotransport controls Na-K-ATPase to a certain degree (Greger et al., 1984c).

Silva et al. (1983) suggest that, upon stimulation of Na-K-ATPase by dibutyryl cAMP and theophylline, the Na-K-ATPase may be covalently modified or change membrane location to increase its activity. In contrast, Shuttleworth and Thompson (1980) suggest the possibility of 'latent sites' of Na-K-ATPase which may be hidden or inactive due to their observations of increased ouabain binding and oxygen consumption without an increase in Na-K-ATPase activity upon cAMP stimulation, indicating the presence of additional Na-K-ATPase. Ouabain binding indicates the number of functional Na-K-ATPase units in the membrane. Shuttleworth and Thompson (1980) suggest that these 'latent sites' may be activated by increased intracellular sodium concentrations.

cAMP and theophylline stimulate the Na-K-2Cl cotransporter, indirectly stimulating Na-K-ATPase (Gregor et al., 1984c) through an increase in intracellular sodium via the Na-K-2Cl cotransporter. As the cotransporter increases the intracellular sodium concentration, Na-K-

ATPase is stimulated to pump the sodium back to the blood. Evidence for this is illustrated by the increased rate of rise of intracellular sodium concentration in the presence of 0.05 mM cAMP and 0.25 mM theophylline in cells blocked by 10^{-4} M ouabain (Shuttleworth and Thompson, 1980). If cAMP and theophylline directly stimulated Na-K-ATPase, presumably little or no increase in intracellular sodium would have resulted as Na-K-ATPase transports sodium out of the cell (Shuttleworth and Thompson, 1980). It is important to note that in incubated tissue preparations, intracellular sodium concentrations increase more in the presence of cAMP and theophylline than in their absence.

The purpose of the epithelial cell in the dogfish rectal gland, as previously mentioned, is to secrete a salt solution which is isoosmotic or slightly hyperosmotic with respect to seawater. It has been established that potassium and chloride are being actively transported into the cell as a result of dissipation of the sodium gradient (Silva et al., 1977)(Silva and Myers, 1986). Sodium is pumped out of the cell, leaving behind chloride and potassium. These ions must also have passage out of the cell and do so through the chloride channels and potassium channels, respectively.

1.5.6 Chloride channel

Chloride channels are located in the apical membrane (Greger et al., 1985) of the epithelial cell. These channels provide a passage for passive diffusion of chloride down an electrical gradient (Silva et al., 1977) into the lumen. There appear to be two types of chloride channels located in the apical membrane. One channel is "large", producing greater currents than a "small" channel as seen through excised membrane patches. The small channels seem to have the ability to remain open longer than the large channels (Greger et al., 1987). The probability of the channel being open increases with a positive voltage or depolarization allowing chloride to pass through. The large channels are extremely permeable to chloride when in the open state and impermeable to sodium (Greger et al., 1987). In contrast to the large channels, the small channels displayed greater activity at a negative voltage as opposed to a positive voltage (Gögelein et al., 1987). Similar to the large channel, the small channel is permeable to chloride and impermeable to sodium as illustrated by a lack of channel activity when a NaCl bath solution was replaced by a Na-gluconate solution. Because channel activity in both large and small channels was constant when bathed in NaCl and KCl, it is assumed that both channels are also impermeable to potassium. (Gögelein et al., 1987).

Chloride moving down a concentration gradient across the apical

membrane produces a lumen negative potential difference needing only a force of 24 mV (Greger and Schlatter, 1984a). Greger et al. (1984c) report that stimulation with a combination of 10^{-4} M dbcAMP, 10^{-4} M adenosine, and 10^{-6} M forskolin produces the previously mentioned lumen negative potential difference. They also found that there is a drop in luminal membrane resistance indicating an increased permeability to chloride. Cyclic AMP appears to be able to stimulate the chloride channel as the chloride concentration within the cell falls quicker during furosemide inhibition of the Na-K-2Cl cotransport in the presence of cAMP than in its absence (Greger et al., 1984c). The stimulated channel is open to allow chloride to pass out depleting intracellular chloride levels.

Chloride channels appear to be present only in stimulated rectal gland tubule segments (Greger et al., 1985). In 114 experiments, only one chloride channel was found in a nonstimulated segment. In contrast, thirty-seven channels were found in stimulated segments.

1.5.7 Potassium channel

Just as chloride passes out of the cell through an apical chloride channel, potassium passes out of the cell through a basolateral potassium channel (Greger and Schlatter, 1984a). The potassium concentrations are greater inside the cell than outside, allowing

potassium to passively diffuse through the membrane. It is estimated that each cell contains approximately 2,000 potassium channels (Greger et al., 1987).

The Na-K-2Cl cotransporter and Na-K-ATPase, then, serve to transport potassium from the blood to the intracellular environment, and the potassium channel recycles it back to the blood. When the rectal gland tubule segments were stimulated with 5 x 10^{-6} M forskolin, 5 x 10^{-4} M adenosine, and 5 x 10^{-4} M dibutyryl cAMP (dbcAMP) to secrete NaCl, patch recorded channel currents directed upwards, indicating potassium was passing out of the cell (Greger et al., 1987).

Greger et al. (1987) found that the potassium channel is impermeable to sodium due to data showing a lack of negative currents at voltages up to -90 mV when the bath was filled with NaCl. They also found that these channels tend to open and close quickly and determined that the channel has four functional states: one open state and three closed states. Greger et al. (1984c) found that the basolateral potassium conductance greatly exceeds the luminal conductance for chloride.

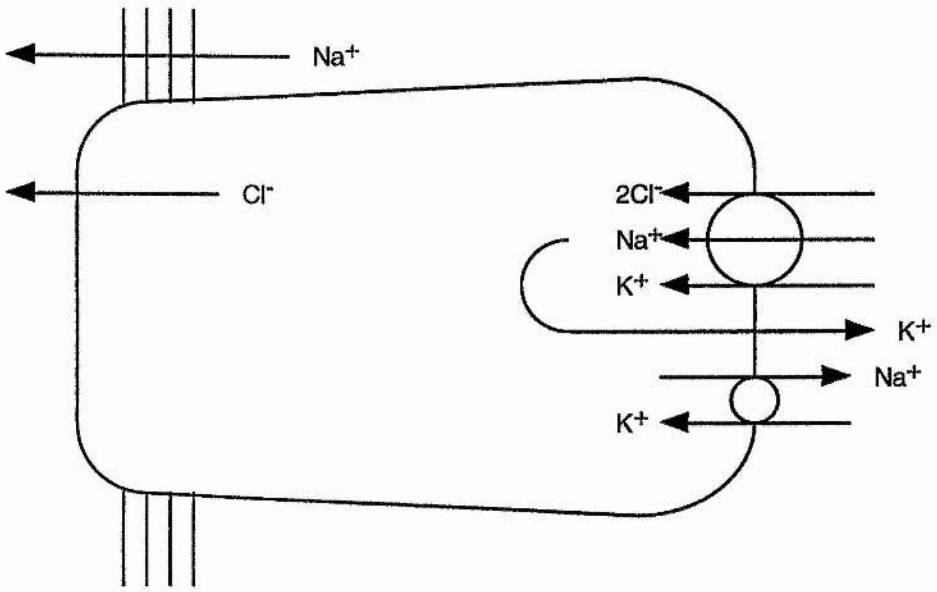
Gögelein et al. (1987) investigated many possible inhibitors of potassium channel activity. Elevated intracellular calcium levels of 10^{-3} M produce marked inhibition of the potassium channels with lesser

inhibitions also seen at 10^{-4} M and 10^{-5} M calcium. Potassium channel activity is also effected by pH. Activity is reduced under acidic pH conditions and increased under alkaline pH conditions. Barium is able to completely inhibit potassium channel activity at levels of 2-5 x 10^{-3} M as does tetraethylammonium (TEA^+) at 20 x 10^{-3} M. Quinidine and quinine produce complete inhibition of channel activity at a concentration of 10^{-6} M. Lidocaine effectively inhibits the channel at 10^{-5} M as does Cs^+ at levels of 5-10 x 10^{-3} M and Rb^+ at 30 x 10^{-3} M.

Na-K-2Cl cotransport, Na-K-ATPase, chloride channels, and potassium channels are all working together to maintain the delicate balance between the internal fluids of the dogfish and its environment. To put these four processes together, a model for NaCl secretion has been developed by Greger and Schlatter (1984a), illustrating the movements of sodium, potassium, and chloride across the cell membrane. To summarize, sodium, potassium, and chloride are carried into the cell via the basolateral Na-K-2Cl cotransporter (Figure 1.12). Sodium is returned to the blood by active transport through the basolateral Na-K-ATPase to maintain the gradient for sodium entry into the cell while potassium is pumped into the cell in exchange . Potassium diffuses out of the cell through basolateral potassium channels due to a high intracellular potassium concentration and low potassium

Figure 1.12

Figure 1.12 Mechanism for chloride secretion and epithelial sodium transport in the epithelial cell of the rectal gland of *Scyliorhinus canicula* and *Squalus acanthias*.


Na+
Cl-
2Cl-
Na+
K+
K+
Na+
K+

concentrations outside the cell. The chloride passes through apical chloride channels down a concentration gradient and into the lumen with sodium following passively through paracellular pathways.

Much work has been devoted to identifying stimulators and inhibitors of the rectal gland salt secretory mechanism. Various techniques have been used to test the effects of these substances on the epithelial transport of the rectal gland. Some of these techniques include isolated perfusion, binding assays, and the culturing of isolated epithelial cells. Each of these methods has provided insight into identifying various substances as stimulators or inhibitors of the transport and the mechanism by which they operate.

1.6 Stimulators

1.6.1 Vasoactive intestinal peptide (VIP)

Various peptides have been of particular interest in stimulation of chloride secretion. VIP potently stimulates secretion rates in the isolated perfused rectal gland of *Squalus acanthias* although apparently not in *Scyliorhinus canicula* (Shuttleworth and Thorndyke, 1984). Silva et al. (1985) estimate the number of VIP receptors per cell to be 4,024 $\pm$ 425 via ^{125}I-VIP binding in isolated rectal gland cells of *Squalus*

acanthias. Immediate stimulation occurred upon infusion of 10^{-6} M VIP increasing secretory volume from 0.5 ml/hr/gram wet weight to 5.5 ml/hr/gram wet weight. The concentration of the chloride in the duct fluid also increased. The stimulation was measureable from 10^{-9} M to 10^{-6} M VIP (Stoff et al., 1979). Shark rectal gland cultures show a characteristic stimulation when exposed to 10^{-7} M VIP. An immediate rise in short circuit current (I_{sc}) to a peak is followed by a gradual, sloping decline (Karnaky et al., 1991; Valentich, 1991). It is interesting to note that VIP can stimulate the cultured epithelium only from the basolateral membrane and is ineffective when applied to the apical membrane (Karnaky et al., 1992).

VIP stimulates cAMP activity through activation of adenylate cyclase (Stoff et al., 1979). Additions of VIP cause intracellular cAMP levels to rise in a dose-dependent manner. The addition of theophylline along with VIP served to augment the increase in cAMP levels. Theophylline is also able to stimulate chloride secretion and enhance intracellular cAMP concentration on its own (Silva et al., 1977; Stoff et al., 1979).

1.6.2 Rectin

Rectin, a peptide isolated from *Scyliorhinus canicula* intestinal

extracts, produced potent stimulation in the perfused *Scyliorhinus* rectal gland (Shuttleworth and Thorndyke, 1984). Rectin also produced increases in ouabain-sensitive oxygen consumption in rectal gland slices, indicating that rectin may be stimulating the cell by way of increased sodium pump activity.

1.6.3 Tachykinins

Two members of the tachykinin family, scyliorhinin I and II, have been isolated (Conlon et al., 1986). Synthetic scyliorhinin II was able to produce stimulation in the isolated perfused rectal gland of *Scyliorhinus canicula* in a dose response manner from 10^{-12} M to 10^{-6} M (G. Anderson, Gatty Marine Lab, University of St. Andrews, St. Andrews KY16 8LB, UK personal communication). The mean chloride clearance rates increased 2.5-fold above basal levels upon perfusion of 10^{-6} M scyliorhinin II while 10^{-9} M scyliorhinin II produced a 2-fold increase with 10^{-12} M producing little stimulation. Scyliorhyinin I and II have not been tested on cultured rectal gland epithelial cells.

It has been suggested that scyliorhinin II and rectin my be identical hormones , although a sequence for rectin was never obtained to confirm this (G. Anderson, personal communication).

1.6.4 Urotensin II

Urotensin II (U II) has been shown to increase blood pressure and pulse pressure in a concentration-dependent manner upon injection of synthetic dogfish U II using in vivo studies with a maximum increase seen at 1.0 nmol (Hazon et al., 1993). In contrast, shark U II did not appear to have an effect on rectal gland secretion rate in perfused in vitro rectal gland (*Scyliorhinus canicula*) preparations (Anderson, personal communication).

1.6.5 Natriuretic peptides

A family of natriuretic peptides has now been identified in various vertebrates and some individual peptides cause potent stimulation in the dogfish rectal gland has been isolated. This family includes four different peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and ventricular natriuretic peptide (VNP) (Figure 1.13). CNP is the only natriuretic peptide to be isolated from the dogfish *Squalus acanthias* (Schofield et al., 1991) and *Scyliorhinus canicula* (Suzuki et al., 1991).

ANP is a secretagogue which has received much attention. Karnaky et al. (1990) report increased short-circuit current (I_{sc}) in shark rectal gland cultures after exposure to 10^{-7} M atriopeptin III (AP-III);(rat synthetic). Stimulation occurs in a dose dependent manner

Figure 1.13

Figure 1.13 Similarities contained in the amino acid sequences in natriuretic peptides of various vertebrates.

Homological sequences between various natriuretic peptides. Dashes represent deleted amino acids (from Takei, 1993).

Atrial Natriuretic Peptide

```
Eel              S K S S S P C F G G K L D R I G S Y S G L G C N S - R K
Bullfrog               S S D C F G S R I D R I G A Q S G M G C - G - R R F
Man           S L R R S S - C F G G R M D R I G A Q S G L G C N S F R - Y
Rat           S L R R S S - C F G G R I D R I G A Q S G L G C N S F R - Y
```

Brain Natriuretic Peptide

```
Fowl             M M R D S G - C F G R R I D R I G S L S G M G C N G S R K N
Pig    S P K T M - R D S G - C F G R R L D R I G S L S G L G C N V L R R Y
Dog    S P K - M M G K S G - C F G R R L D R I G S L S G L G C N V L R D Y
Cattle   P K - M M R D S G - C F G R R L D R I G S L S G L G C N V L R R Y
Man    S P K - M V Q G S G - C F G R K M D R I S S S S G L G C K V L R R H
Rat    S - K - M A H S S S - C F G Q K I D R I G A V S R L G C D G L R L F
```

C-Type Natriuretic Peptide

```
Dogfish          G P S R G - C F G V K L D R I G A M S G L G C
Eel              G W N R G - C F G L K L D R I G S L S G L G C
Killifish        G W N R G - C F G L K L D R I G S M S G L G C
Bullfrog         G Y S R G - C F G V K L D R I G A F S G L G C
Fowl             G L S R S - C F G V K L D R I G S M S G L G C
Man              G L S K G - C F G L K L D R I G S M S G L G C
```

Ventricular Natriuretic Peptide

```
Eel   K S F N S - C F G T R M D R I G S W S G L G C N S L - K N G T K K K I
      F G N
```

from 10^{-9} M to 10^{-6} M. Removal of chloride$^-$ from the apical and basolateral baths produced a drop in I_{SC} indicating that chloride was the ion being transported. Stimulation by 10^{-7} M AP-III produces an initial rise in I_{SC} similar to VIP, but the rise is then followed by oscillations before a steady state is reached (Karnaky et al., 1991). In contrast, Silva et al. (1987) report that atriopeptins may not act directly to stimulate chloride secretion, but they act indirectly by the release of VIP which acts as a neurotransmitter. This conclusion was drawn as a result of data showing neurotransmitter inhibitors including 1) the presence of procaine (10^{-2} M) in rectal gland perfusate; 2) perfusion with high magnesium (9.5×10^{-3} M), low calcium (5×10^{-2} M) solutions; and 3) perfusion with the calcium channel blockers diltiazem (5×10^{-5} M), nifedipine (10^{-6} M), or verapamil (10^{-4} M) block the effects of atrial natriuretic peptide II (ANP II) and the release of VIP into the venous effluent of the perfused glands in response to addition of cardiac peptides (Silva et al., 1987).

The idea of ANP using VIP as a neurotransmitter is brought into question due to conflicting data (Karnaky et al., 1990; Karnaky et al., 1991). First, the I_{SC} produced upon stimulation of shark rectal gland cultures are not the same. As previously mentioned, ANP produces

characteristic oscillations and then steady state secretion whereas VIP produces a large initial stimulation followed by decline. Second, atriopeptin I (AP-I);(rat synthetic) can activate chloride secretion in cultures from the apical and basolateral sides (Karnaky et al., 1990; Karnaky et al., 1991) as opposed to strict basolateral stimulation by VIP. Finally, the time between addition of AP-I or AP-III and stimulation was equal for both apical and basolateral additions, indicating that the peptide was not diffusing through paracellular pathways to stimulate chloride secretion from the basolateral side. If this were the case, a time difference would be expected (Karnaky et al., 1990).

ANP also differs from VIP in that it elevates guanosine 3', 5'-monophosphate (cGMP) levels. After addition of 10^{-7} M AP-III, cGMP levels in cultured cells rose approximately five times its previous values (Karnaky et al., 1990). This indicates ANP may be using cGMP as a second messenger for chloride secretion.

CNP, an ANP-like hormone, has been identified from the heart of *Squalus acanthias* (Schofield et al., 1991) and *Scyliorhinus canicula* (Suzuki et al., 1991). Shark CNP illicits a strong chloride secretion response in isolated perfused *S. acanthias* rectal glands in a dose response manner beginning at 10^{-10} M, with chloride secretion being similar to that of VIP (approximately 600 microEq/h/g) at

concentrations below 5 x 10^{-8} M (Solomon et al., 1992). Forrest et al. (1992) isolated a shark CNP which potently stimulates chloride secretion in the perfused shark rectal gland compared to mammalian ANP.

Karnaky et al. (1992) found that killifish CNP is able to stimulate chloride secretion in the cultured shark rectal gland at concentrations as low as 10^{-10} M. Similar to ANP, CNP is also able to stimulate from the apical and basolateral side of the cell. In addition, killifish CNP produced a rise in cGMP levels indicating cGMP as a possible second messenger for killifish CNP stimulation.

1.6.6 Forskolin

Another potent stimulator of chloride secretion is forskolin. Forskolin at 10^{-6} M raises chloride secretion in perfused rectal glands 12.8 times above its resting values by activating adenylate cyclase (Kelley et al., 1990). Forskolin (10^{-6} M) was able to stimulate a rise in cAMP levels fifty-eight times the resting values after 80 min of incubation. Forskolin (10^{-4} M) was able to stimulate adenylate cyclase activity approximately sevenfold over resting values. Differences seem to be developing between the stimulators as to which second messenger they stimulate. The cardiac peptides apparently make use of cGMP as opposed to cAMP which is used by VIP and forskolin.

Simpson and Sargent (1985) report maximal stimulation of oxygen uptake in rectal gland slices incubated in elasmobranch ringer and 8 x 10^{-5} M forskolin. The stimulations were approximately 6.6-fold over resting values in the dogfish shark *Scyliorhinus canicula.*

1.7 Inhibitors

Inhibitors are important to the study of the mechanism of chloride secretion because they can illustrate the steps involved in chloride secretion and allow for identification of particular components of secretion such as Na-K-2Cl cotransport and Na-K-ATPase. Experimentally, naturally occurring endogenous inhibitors inhibitors indicate the degree to which a membrane is stimulated and identify the stability of a particular membrane after a stimulation has occurred.

1.7.1 Somatostatin

Somatostatin is a potent inhibitor of VIP-induced stimulation at a concentration of 1.4 x 10^{-7} M (Stoff et al., 1979). Interestingly, no effect was measured on chloride secretion alone. Upon removal of somatostatin, the VIP-induced stimulation returned indicating that the perfused gland was still viable.

In further experiments, Silva et al. (1985) noticed somatostatin

produced no effect on ^{125}I-VIP binding in perfused rectal glands, but it did completely inhibit VIP-induced cAMP accumulation. Again, somatostatin had no effect on the unstimulated cell. Because somatostatin does not inhibit VIP binding, but does inhibit VIP-induced cAMP accumulation, it appears that somatostatin may exert its inhibitory effects on adenylate cyclase (Silva et al., 1985). As previously mentioned, VIP may stimulate cAMP accumulation via adenylate cyclase (Stoff et al., 1979). Due to data showing inhibition of dbcAMP stimulation (Silva et al., 1985), somatostatin may also inhibit a mechanism at some point distal to cAMP production.

Forskolin, a direct activator of adenylate cyclase (Kelley et al., 1990), is also inhibited by somatostatin (Epstein et al., 1992). Somatostatin (10^{-6} M) added to the basolateral surface reduced stimulation by forskolin in cultured dogfish (*Squalus acanthias*) rectal glands by lowering the I_{SC} by 52 $\pm$ 10%. This data support the idea of somatostatin inhibition of adenylate cyclase. In fact, 10^{-7} M somatostatin inhibits basal adenylate cyclase by 37% and 10^{-4} M forskolin stimulated adenylate cyclase by 25% (Kelley et al., 1990).

Solomon et al. (1984b) report the inhibition of chloride secretion by somatostatin in response to volume loading in the isolated perfused rectal gland. After 150 ml of isotonic shark ringer were infused, 4.5 x

10^{-4} M somatostatin markedly inhibited chloride secretion when compared with control values. A vasodilatory response which accompanies volume loading was not inhibited.

1.7.2 Bombesin

Similar to somatostatin, bombesin also inhibits chloride secretion in VIP-stimulated perfused rectal glands. VIP (2 x 10^{-9} M) was perfused for thirty min. followed by perfusion of 8 x 10^{-7} M bombesin followed by a return to VIP perfusion. Bombesin inhibited chloride secretion by 53 $\pm$ 4.6%. Chloride secretion returned to normal after bombesin was removed. Control experiments performed without bombesin showed a normal steady decline in secretion much different from the pattern with bombesin (Silva et al., 1990).

Silva et al. (1990) stimulated chloride secretion in rectal glands by perfusing with 5 x 10^{-5} M dbcAMP and 2.5 x 10^{-4} M theophylline and then inhibited the response by subsequent addition of 8 x 10^{-7} M bombesin. Bombesin produced inhibition of chloride secretion by 42.7 $\pm$ 3.7%. Again, chloride secretion returned to the stimulated rate after removal of bombesin and continued perfusion of 5 x 10^{-5} M dbcAMP and 2.5 x 10^{-4} M theophylline. Nifedipine is a neurotransmitter

inhibiter and calcium channel blocker which inhibits the effects of bombesin but not somatostatin. As a result, nifedipine may suppress the bombesin-stimulated release of somatostatin (Silva et al., 1990).

These data indicate that bombesin has a very similar effect on stimulated chloride secretion as does somatostatin and may exert its inhibitory effects on the rectal gland via the release of somatostatin. In fact, addition of bombesin to unstimulated rectal glands produced a rise in somatostatin in the venous effluent (Silva et al., 1990; Silva et al., 1993) and when the release of somatostatin was inhibited by cysteamine (2.6×10^{-3} M), the inhibitory effects of bombesin was reduced from $53 \pm 4.6\%$ to $37.4 \pm 4.1\%$ (Silva et al., 1990). Epstein et al. (1992) found that bombesin produced no inhibition in stimulated rectal gland cultures. This may be due to the fact that bombesin is not able to stimulate somatostatin release in culture.

1.7.3 Neuropeptide Y

Neuropeptide Y (NPY) is able to inhibit chloride secretion in perfused rectal glands stimulated with VIP. Stimulation was produced by perfusion of 1.67×10^{-9} M VIP for thirty min followed by constant infusion of NPY to the perfusate for an additonal thirty min. NPY (10^{-7} M) was able to reduce chloride secretion by $63.0 \pm 3.4\%$ with secretion

values returning to VIP stimulated levels after removal of NPY (Silva et al., 1993). NPY also inhibited forskolin stimulated chloride secretion in cultured rectal gland cells (Epstein et al., 1992; Silva et al., 1993).

Stimulation via 8-chlorophenylthio-cAMP (8-CPT-cAMP) and dbcAMP and theophylline was also inhibited by NPY (Silva et al., 1993). This indicates a possibility that NPY exerts its inhibitory effects on a site after the generation of cAMP. It must be noted that NPY appears to have no effect on adenylate cyclase.

1.7.4 Bumetanide

Bumetanide is a potent inhibitor of the Na-K-2Cl cotransport system (Palfrey et al., 1984). When bumetanide was perfused into the rectal gland, chloride secretion was decreased along with a drop in adenosine concentration (Kelley et al., 1991). Adenosine is produced in the cell and transported to the extracellular space, where it can bind with adenosine receptors to inhibit chloride secretion (Kelley et al., 1991). Adenosine levels were shown to increase with stimulated chloride secretion.

Bumetanide is able to inhibit the secretory response to volume loading and also secretion due to addition of dbcAMP and theophylline (Solomon et al., 1984b). The inhibition produced by bumetanide was significant although the vasodilatory response produced by the

stimulants was not inhibited.

1.8 Conclusion

Clearly, the dogfish shark rectal gland is an ideal model for study of epithelial sodium transport. Although the teleost chloride cell is an excellent model of ion transport, a variety of cell types, including mucous, pavement, and non-differentiated cells leave chloride cells in the minority, making cell isolation difficult (Karnaky and Kinter, 1977). The rectal gland is nearly a homogenous collection of tubules composed entirely of columnar epithelial cells with homogenous functions (Valentich, 1991). The rectal gland also provides these cells in large numbers, lending it to cell isolation experiments such as tissue culture. Tissue culture is a particularly attractive method of study because it allows for experimentation on an isolated, homogeneous collection of cells undergoing epithelial sodium transport. Valentich (1991) previously developed a technique for culturing rectal gland cells of *Squalus acanthias.* This project seeks to modify Valentich's method or to develop a new technique of rectal gland cell culture to optimize cell yield using the smaller dogfish, *Scyliorhinus canicula.* The recent isolation of homologous elasmobranch endocrine hormones such as CNP (Suzuki et al., 1991), VIP (Dimaline, 1987), and the tachykinins: scyliorhinin I and II (Conlon et al., 1986) are ideal for experimentation

on the cultured epithelial cells..

The purpose of this project is three fold: 1) to establish a method for preparing epithelial cell cultures using the rectal gland of *Scyliorhinus canicula* ; 2) to optimize the growth of these cells; and 3) to test the sensitivity of these cells to endogenous peptides such as CNP, VIP, and scyliorinin I and II by measuring the stimulation of intracellular cGMP and cAMP concentrations.

2.0 Materials and Methods

2.1 Supply of fish

Dogfish (*Scyloirhinus canicula*) were obtained from fishermen off the coasts of Oban, Scotland and Bangor, Wales. They were transferred to Gatty Marine Laboratory and kept in through flowing tanks in seawater maintained at ambient temperatures.

2.2 Fixing and staining tissue

The dogfish were then removed from the tank and killed humanely by destruction of the central nervous system. A ventral incision was made approximately 10 cm anterior to the anus and the incision was extended 5 cm anteriorly and posteriorly. The flaps of skin were retracted to expose the rectal gland lying at the posterior end of the spiral intestine.

The rectal gland was removed by cutting the rectal gland vein, duct, artery, and any connective tissue attached to the gland. The gland was fixed, sectioned, and stained as in Tables 2.1-2.4.

2.3 Sterile shark Ringer

The procedure for preparing sterile shark Ringer was carried out in a 1.0 litre beaker and with deionized (Milli-Q) water. The following solutions were added to approximately 300 ml of water for preparation of 1.0 litre of Ringer.

Table 2.1

Table 2.1 Technique for fixing of rectal gland tissue.

Fixing tissue

A. The gland was placed in a universal tube containing approximately 15 ml of Bouin's fixative for 10 min.

B. A transverse section was made through the middle of the gland and the gland was placed back into the Bouin's for and additional 5 hours.

C. The gland was removed from the fixative and placed in a series of alcohol and chloroform solutions (Table 2.2).

D. Following the chloroform, the gland was placed in three different baths of paraffin wax under a vacuum. The first two baths were for one hour each and the third was left for three hours.

E. The tissues were placed in cold water to allow the wax to solidify.

Table 2.2

Table 2.2 Time intervals of alcohol and chloroform applications involved in the fixing of the rectal gland.

Solution	Time (hr)
75% alcohol	overnight
75% alcohol	2
96% alcohol	1
96% alcohol	2
absolute alcohol	1
absolute alcohol	1
chloroform	1
chloroform	overnight

Table 2.3

Table 2.3 Technique for sectioning rectal gland tissue.

Sectioning tissue

A. The sections were cut to 6-8 microns, using a rotary microtome.

B. The sections were carefully placed on the surface of a cold water bath.

C. A slide was dipped underneath the sections to position the sections in the middle of the slide while keeping the sections as flat as possible.

D. The slide was allowed to dry in an incubator.

E. To remove the paraffin, the slide was placed in Xylol for 1-2 min.

Table 2.4

Table 2.4 Solutions and procedure for modified Masson's Trichrome Stain.

(from Masson, 1929)

Staining tissue

Solutions

1. Celestine Blue: 0.5 g of dye was added to 100 ml of 5% iron alum and boiled for 3 min. When cool, the solution was filtered and 14 ml of glycerine added.
2. Mayer's Haemalum: 1 g of Haematoxylin dye was dissolved in 10 ml of absolute alcohol for approximately 10 min. 1 L of distilled water along with 50 g of potassium alum (aluminium potassium sulphate) and 0.2 g of sodium iodate were added to the above and allowed to stand overnight. 50 g of chloral hydrate were added along with 1 g of citric acid and boiled for 5 min. The solution was cooled and filtered and stored at room temperature.
3. Yellow Mordant: 160 ml of saturated picric acid in 96% alcohol was added to 0.40 g of Orange G and 0.40 g of Lissamine Fast Yellow was added to distilled water. The two solutions were combined and stored at room temperature. To prepare a working solution, dilute 30 ml of stock with 70 ml of 75% alcohol.
4. Ponceau Acid Fuchsin: 2.00 g of Ponceau 2R (Ponceau de Xylidine) and 1.00 g of Acid Fuchsin were added to 200 ml of distilled water and 3 ml of acetic acid.
5. 1% aqueous phosphomolybdic acid
6. 1% Aniline Blue or Light Green in 1% acetic acid

Procedure	Time (min)
1. Stain with Celestine Blue	10
2. Rinse in distilled water/stain with Mayer's Haemalum	10
3. Rinse in running tap water	5
4. Place in Yellow Mordant	3
5. Wash with running tap water until the tissue is almost colorless	2-10
6. Stain with Ponceau Acid Fuchsin	5
7. Rinse briefly with tap water and mordant in 1% phosphomolybdic until connective tissue is free of red dye	5-20
8. Stain with Aniline Blue or Light Green	2
9. Rinse with 1% acetic acid followed by 96% alcohol	1-2
10. Dehydrate with absolute alcohol, clear in xylol followed by mounting in DePeX.	

11. The staining is as follows: Nuclei-black; muscle, red blood cells, tubules-red; collagen (connective tissue), mucin-blue.

Solution (Molarity)	Volume (ml)
4.004 M NaCl	60.0
2.398 M KCl	3.0
0.1 M $CaCl_2$	50.0
0.99M $MgCl_2$	5.0
0.459M $NaHCO_3$	50.0
0.0876 M Na_2HPO_4	5.0
0.099 M Na_2SO_4	5.0
3.59 M Urea	100.0
0.599 M TMAO	100.0
0.5% Phenol red	0.25

Glucose was then added to 1% w/v. All of the above solutions were mixed except for the 0.1 M $CaCl_2$ in a 1 liter flask and the volume was brought up to 800 ml and the solution was gassed with 95% O_2:5% CO_2. The pH should be brought to between 7.4 to 7.6 judged by using a pH meter. The 0.1 M $CaCl_2$ was then added slowly with constant stirring to avoid precipitation. The volume was brought up to 1 litre and sterilized with a 0.22 μm filter and stored at 4° C. The Ringer was gassed before use to ensure a pH of 7.4 to 7.6.

2.4 Sterile culture medium

The culture medium was prepared by adding the following compounds to approximately 250 ml of Dulbecco's MEM/nut mix Ham's F-12 with L-glutamine and 15 mM HEPES in a 1 litre beaker with stirring.

Compound	Final molarity (mM)	mass (g)
NaCl	100	2.922
$NaHCO_3$	21	0.882
$CaCl_2$	3.9	0.288
(dissolve $CaCl_2$ in 25ml of Dulbecco's MEM/nut mix Ham's F-12)		
MgCl2	2.5	0.254
Urea	300	9.009
Trimethylamine oxide	150	8.368

After the above compounds were added and dissolved, 25 ml of Nu-Serum (Stratech Scientific, 61-63 Dudley Street, Luton, Beds), 5 ml of ITS^+ (Stratech Scientific, 61-63 Dudley Street, Luton, Beds), and 5000 units/ml of penicillin/ 5 mg/ml streptomycin were added. The pH was adjusted to 7.6 with and the volume was brought up to 500 ml with Dulbecco's/Ham's F-12. The medium was sterilized by passing through a 0.22 μm filter and stored at 4^0 C.

2.5 Modification of Valentich's rectal gland tissue culture technique-Method 1 (Valentich, 1991)

The technique developed by Valentich (1991) for *Squalus acanthias* was adapted to prepare isolated rectal gland epithelial cells from *Scyliorhinus canicula*. Dogfish were removed from the tank and humanely killed. The rectal gland was exposed as previously described (section 2.2). Using a pair of fine scissors, the gland was removed cutting away as much connective tissue as possible without damaging the gland.

Once the gland was removed from the dogfish, it was placed in a 30 ml universal tube with approximately 15 ml of ice cold sterile shark Ringer and placed on ice. All subsequent procedures were conducted under sterile conditions.

Under a flow hood, a glass petri dish was placed on an ice pack. The dish was filled with approximately 5 ml of ice cold Ringer. The rectal gland was placed in the petri dish and was sliced transversely into 1mm slices with a scalpel. The two ends of the gland were discarded as they typically contain large amounts of connective tissue (Valentich, 1991).

The slices were transferred using forceps to a petri dish on ice and cut into pieces less than 1 mm^3. Once this was completed, the small pieces were minced with two scalpels using criss-cross action (Valentich,

1991) for approximately 5 min. If the tissue became dry and sticky, a drop or two of Ringer was added.

After mincing, 5 ml of Ringer was added and dispersed 7 times using a 5 ml syringe and a steel needle. The mince was transferred to a centrifuge tube and an additional 5 ml of Ringer added to the petri dish to wash any remaining pieces. This process was repeated and the whole process was performed on ice.

The mince was washed 3 times with ice cold Ringer and allowed to settle each time by gravity. By the third rinse, the Ringer would normally appear clear. The mince was centrifuged for 30 seconds at 1000 rpm at 4° C. The supernatant was discarded and the pellet resuspended in 5 ml of Ringer conaining 2 mg/ml collagenase-D (Boehringer Corporation Ltd., Mannheim House, Bell Lane, Lewes, East Sussex) and the tube gassed with 95% O_2:5% CO_2. The tube was placed on a rocking platform for 45 min and checked periodically to ensure no tissue clumping occurred.

After 45 min, the mince was vigorously dispersed 10 times (careful not to cause frothing) and the tube was gassed again with 95% O_2:5% CO_2 and rocked for an additional 45 min making sure no clumping occurred. If clumping did occur, the tube was shaken to disperse the mince.

The tube was removed from the rocking platform, the mince

dispersed 10 times (as above), and the tissue allowed to settle for approximately 30 seconds. The supernatant was saved using a 10 ml syringe fitted with a sterile needle and placed in a centrifuge tube. Ringer (6-7 ml) was added to the mince, dispersed 5 times, and allowed to settle for 30 seconds. The supernatant was placed in the same centrifuge tube. More Ringer was added and the process repeated once. Only the supernatants were saved, leaving the centrifuge tube containing isolated rectal gland tubules.

The combined supernatants were centrifuged for 45 seconds at 225g at 4° C. The supernatant was discarded and the pellet was resuspended in approximately 5 ml of ice cold Ringer and centrifuged for 45 seconds at 1000 rpm and 4° C. This washing procedure was repeated two more times.

The final pellet was suspended in 3 ml of medium and plated (section 2.8).

2.6 Basic perfusion technique-Method 2

Dogfish were removed from the tank and given an injection of 1000 units of heparin via the caudal sinus and allowed to swim freely for 10-15 min. The dogfish were again removed from the tank and humanely killed as above.

The ventral surface of the dogfish was exposed as in section 2.1

and all procedures were carried out under sterile conditions in a flow hood (either Bassaire or Gelaire);(Bassaire Ltd., Southampton, Hampshire, GB; Gelaire Luminar Air Flow Class 100, Gelman Instruments, Northampton, England).

The intestine was tied off with surgical thread approximately 3.5 cm from the rectal gland and the intestine cut posterior to the thread. Using small vanna scissors (curved, 3 mm cutting length; John Weiss and Son Ltd., Milton Keynes, UK), a small cut was made in the rectal gland vein approximately 1.5 cm from the gland itself.

A sterile intravenous cannula (OD-0.63 mm, size 2FG; Portex Limited, Hythe, Kent, England) was prepared previously containing ice cold, calcium-free Ringer. The end of the cannula was cut to a point, and the cannula was filled with sterile calcium-free Ringer to ensure no air blocked the perfusion. The cannula was carefully inserted into the rectal gland vein via the cut and slowly slid to approximately 2-3 mm from the gland in order to maximize perfusion of the gland. Once the cannula was in place, a second piece of surgical thread was tied around the vein and cannula. The first piece of surgical thread, located on the intestine, was also then tied around the cannula to maintain the cannulae in position.

Using scissors, the gland was separated from connective tissue and carefully removed from the dogfish. 0.1-0.2 ml of calcium-free Ringer

was slowly passed through the gland using the syringe to help ensure a good perfusion. All procedures after this stage were perfomed under sterile conditions.

The cannula was removed from the syringe while ensuring no air was introduced into the apparatus. The cannula was then attached to the perfusion apparatus (Figure 2.1). The perfusions were carried out under gravity as follows: 1) 5 ml of calcium-free Ringer + 1 mM ethyleneglycol-bis-(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), 2) 5 ml of collagenase D (2 mg/ml in Ringer containing100 μM calcium), and 3) 5 ml of calcium-free Ringer. The approximate flow rate was 1 ml every 5 min.

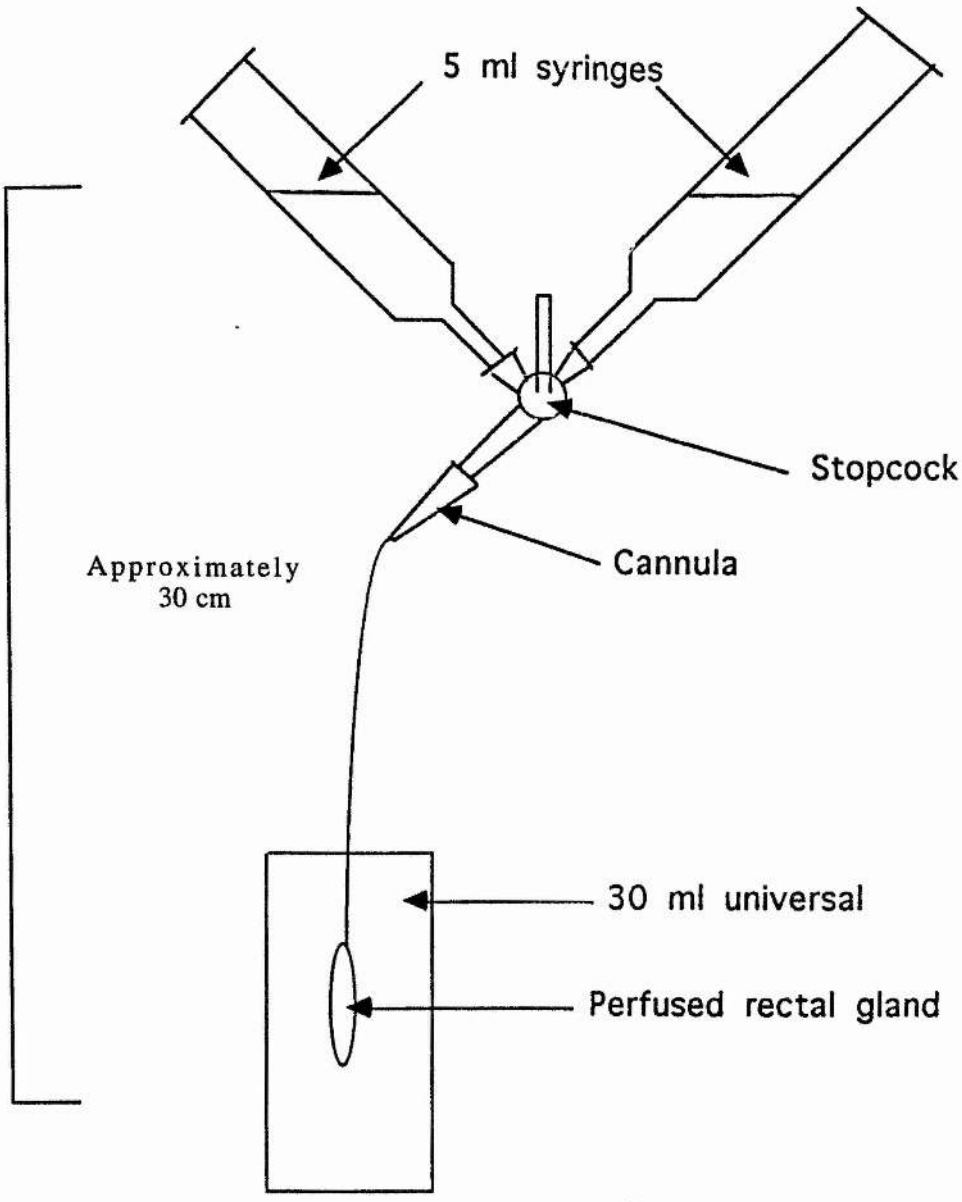
After all the perfusions were completed, the gland was removed from the cannula and transferred to a glass petri dish, containing 2-3 ml of ice cold Ringer. The petri dish was resting on an ice pack to ensure the gland remained cold.

Using a scalpel with a No. 20 surgical blade (Swann-Morton, Sheffield, England), the two ends of the rectal gland were removed. The gland was then transferred to a second glass petri dish, containing no Ringer and placed on the ice pack.

The gland was cross-sectioned, using the surgical blades, into 2-3 mm thick slices. These slices were scraped by placing each slice on its side and making a radial cut to allow the slice to be opened up and laid,

Figure 2.1

Figure 2.1 Perfusion apparatus.


5 ml syringes
Stopcock
Cannula
Approximately
30 cm
30 ml universal
Perfused rectal gland

connective tissue capsule side down, on the dish. The tissue was then carefully scraped free of the capsule by stabilizing one end of the strip with one blade and scraping with the second blade. Once the tissue was scraped free, it was minced, using criss-cross action, for 5 min.

Sterile Ringer (7 ml) was added to the mince followed by 7 dispersions using a 10 ml syringe with a sterile needle. The suspension was then passed through two layers gauze. The dish was washed with 4 ml of Ringer which was again passed through gauze.

The mince was then centrifuged in a coolspin (MSE) at 225g for 1.5 min at 4° C. The supernatant was aspirated and the pellet resuspended and washed in 7 ml of ice cold Ringer. The suspension was centrifuged again at 225g for 1.5 min at 4° C. The supernatant was aspirated and the pellet resuspended in 3 ml of sterile medium.

The above procedure was arrived at only after numerous experiments which used the following variations: 1) calcium concentration (0.1 mM or 5.0 mM), 2) mincing or scraping, 3) number of dispersions, 4) Worthington's collagenase (2mg/ml), 5) mince time, 6) omitting gauze, 7) number of washes, 8) incubation temperature, 9) and solutions perfused, including Ringer, medium, collagenase, and1 mM sodium nitroprusside (SNP).

2.7 Collagen coating of 35-mm and 96-well plates

35-mm plates and 96-well plates were collagen coated with Type 1 collagen (Sigma, St. Louis, MO, USA) 1.4 ml/plate and 30 µl/well, respectively. The collagen was allowed to dry overnight and 1.5 ml of medium for the 35-mm plates and 150 µl of medium for the 96-well plates was added and left at room temperature for one hour before removing the medium just prior to the plating of tubules.

2.8 Plating

The final pellet, suspended in 3 ml of sterile medium (Dulbecco's MEM/nut mix Ham's F-12), was plated in various manners (all plates were coated with Type 1 collagen): 1) suspension could be plated 1.5 ml/plate on sterile, 35-mm, culture dishes (Corning Glass Works, Corning, N.Y., USA) with or without a sterile cover-slip (these dishes were not previously treated by the manufacturer) and 2)150 µl/well on 96-well plates (Bibby Sterilin, Stone, Staffs, U.K.).

The dishes were placed in a sterile, plastic container and gassed with 95% air/5% CO_2. The container was sealed and the cultures were incubated on a level surface at the desired temperature.

The cultures were left unmoved for 48-hours after which time half of the medium was removed and replaced with an equal amount of fresh medium. After the first change, the full amount of meduim was

changed once every two days and up to a maximum of twenty days.

2.9 Cell counting

Cell counting was accomplished through the use of a Coulter Counter (Model ZM with Coulter Channelyzer Model 256, Coulter Electronics LTD., Beds, England). The cultures were typically 10-14 days old.

2.9.1 Counting epithelial cells in 96-well plates

The medium was removed from each well and 100 µl of 1% trypsin + 2 mM EDTA + calcium- and magnesium-free Ringer were added to each well. The well was placed on a shaker for approximately 20 min or until the cells detached from each other into single cells. Each well was neutralized with 100 µl of Ringer containing 0.5% (v/v) fetal calf serum. The wells were dispersed seven times and the cell solution was removed and placed in a Coulter vial containing 14.8 ml of Ringer. The cells were then counted on the Coulter Counter.

2.10 Preparation of Ethidium Bromide/ Acridine Orange (EBAO)stock solution

1. 50 mg of ethidium bromide and 15 mg of acridine orange were dissolved in 1 ml of 95% ethanol.
2. 49 ml of distilled water were added with thorough mixing.

3. The solution was divided in 1 ml aliquots and stored frozen at -20°C.
4. To prepare working strength EBAO solutions add: 1 ml stock to 99 ml of shark Ringer without phenol red.

2.11 Photographs of epithelial cells

Cell cultures were prepared on Type 1 collagen coated cover-slips in 35-mm plates. The medium was removed and replaced with 1.5 ml of Ringer (no phenol red) to wash. The Ringer was removed and replaced with 1 ml of working strength EBAO in clear ringer. The EBAO was allowed to sit for 1.5 min and then removed. Clear ringer was used to wash the cells by applying 1 ml to the dish. Repeat the wash once.

The coverslip was carefully removed from the dish with fine forceps, and the bottom of the coverslip was dried with a tissue. A drop of Ringer was placed on the middle of the slide, and the coverslip was carefully placed, cell side down, onto the slide.

The cells were then observed under phase contrast and fluorescence microscopy.

2.12 cGMP assay

2.12.1 Preparation of reagents for cGMP assay

Acetylation mix: 2:1 Triethlyamine: Acetic Anhydride
Combine these in a glass tube and vortex immediately.

Extracted/neutralized

Ringer buffer: 50 ml of shark Ringer + 50 ml of 6% Perchloric acid (PCA). Neutralized with 3M K_2CO_3 to pH of 6-7 and left on ice for 10-15 min. Centrifuged at 2000 g at 4° C for 15 min.
Supernatants were stored at -20° C for use.

Buffer: 50 mM sodium acetate to pH of 4.75 (2.051g/500 ml) contains 0.5% BSA (2.5g/500ml). Can pH with glacial acetic acid.

Antibody: Combine 1 ml of (1;1000) diluted rabbit anti cGMP antibody with 24 ml of the sodium acetate buffer. (dil: 1:24,000)

Radiolabel: [^{125}I] cGMP (TME) (Approximately 15,000cpm/100 µl in buffer).

Standards: 40 µl 200 µM cGMP stock + 1.96 ml buffer = 4

μ M

100 μl 4 μM cGMP + 2.4 ml buffer = 0.16 μM

(16 pmols/100 μl).

2.12.2 cGMP assay on isolated tubules

2.12.2a Sample preparation

To perform the cGMP assay on isolated rectal gland tubules, the procedure for preparing and isolating rectal gland tubules was followed as described above with the exception of the final suspension of tubules in culture medium. In this procedure, the final pellet was suspended in 2.0 ml of shark Ringer. This preparation will be referred to as the 'tubule suspension'.

All of the following procedures were performed on ice. Hormone concentrations of shark CNP (sCNP), urotensin II, or Scyliorhinin II were prepared as desired. Each hormone was diluted in shark Ringer containing 3 mM 3-isobutyl-1-methyl-xanthine (IBMX). Tubule suspension (50 μl) was added to each RT-30 tube containing 50 μl of hormone prepared for the desired concentration and the reaction was stopped with an equal volume of 6% PCA (100 μl). Once the reaction was stopped, the mixtures were allowed to stand for approximately 10 min before 10-20 μl of 3 M K_2CO_3 was added to neutralize the mixture.

The amount of 3 M K_2CO_3 needed was determined by the amount required to bring the mixtures to a pH of 6-7. After addition of 3 M K_2CO_3, the tubes were immediately vortexed, left on ice for 10-15 min, and then centrifuged (15 min) at 6000 rpm (3180 g). The supernatant was removed and placed in RT 30 tubes and the pellet discarded. If required, the supernatant could be frozen overnight.

The samples were aliquoted by placing 75 µl of sample into each of two tubes for each sample present. Thus 150 µl was removed from each sample. Each 75 µl sample was diluted with 25 µl of deionized water. Acetylation mix (1 µl) was then added to each tube and vortexed.

2.12.2b Standard preparation

The stock was cGMP stock solution (2-5 ml of 0-16 µM) acetylated with 10 µl of acetylation mix and allowed to stand for 30 min at room temperature.

After 30 min, 1:1 dilutions were performed on the standards using extracted shark Ringer buffer. The 0 contained 100 µl of extracted Ringer buffer, 100 µl of [^{125}I] cGMP, and 250 µl of cGMP antibody. The non-specific binding contained 100 µl of [^{125}I] cGMP, 250 µl of cGMP antibody, and 100 µl of 4 µM cGMP. The total binding contained 100

µl of [^{125}I] cGMP. The 0, non-specific binding, and total counts were prepared in duplicate.

From the tube containing 2 pmol/100 µl of stock to the tube containing 0.0019 pmol/100 µl of stock, 100 µl samples were removed and placed in tubes in duplicate. To these standards and to all samples,100 µl of [^{125}I] cGMP and 250 µl of cGMP antibody, in that order, were added into each tube. All tubes were mixed gently and incubated overnight at 4^{0}C.

After incubation, 2.0 ml of 95% ethanol was added to each tube (except for the total counts tubes), vortexed, and allowed to stand for 30 min at room temperature. After 30 min, the tubes were centrifuged (MSE) at 2400 rpm (1320g) for 30 min at 4^{0}C. The resulting supernatant was poured off and the radioactivity of the pellet was counted on a gamma counter (Cobra II Auto-Gamma counter, Canberra Packard, Pangbourne, Berks).

2.12.3 cGMP assay procedure for cultures on a 96-well plate

The sCNP was dissolved in the following solution:

Into 10 ml of ringer was added: 10 mg bacitracin

10 µl 1 mM leupeptin

10 µl 500 µM phosphoramidon

10 µl aprotinin

6.67 mg IBMX

100 µl of the appropriate mix was added to each well and stopped by the addition of 100 µl of ice cold 6% PCA after the desired time period and allowed to sit for approximately 10 min at 4° C before neutralization with 3 M K_2CO_3 to a pH of 6-7. The 96-well plate was centrifuged at 250g for 10 min at 4° C. Aliquots (50 µl) of supernatant were removed and placed in 3 tubes and diluted with an equal volume of deionized water. Acetylation mix (1 µl) was then added to each tube and vortexed. The samples were then allowed to sit for 1-24 hours on ice or overnight at -20° C. The procedure is the same as the previous procedure from this point.

2.13 cAMP assay on isolated rectal gland cells

2.13.1 Preparation of reagents for cAMP assay

[^{3}H]-cAMP (Amersham International plc., Buckinghamshire,Eng.) - 2 µl of stock [^{3}H]-cAMP (30-50 Ci/mmol: 1 mCi/ml) into 5 ml of distilled water.

Specific binding protein - prepared from adrenal glands by the method of Brown et al. (1971) and stored frozen at -20° C.

Binding protein - 2 ml of stock binding protein was diluted with 28 ml of cAMP assay buffer (listed below).

Charcoal mix - 1.0 g of charcoal in 50 ml of water plus 0.1 g of bovine serum albumin. The solution was mixed and allowed to sit for at least 15 min before use. Mixing was continued through the charcoal addition procedure.

Assay incubation buffer
50 mM Tris
4.0 mM EDTA
0.1 mM dithiothreitol
1.0 mM IBMX
pH = 7.8

2.13.2 Sample preparation

The rectal gland isolated tubules were prepared as above (section 2.10.2), and to each RT 30 tube containing 100 µl of hormone was added 100 µl of tubule suspension (suspended in shark Ringer). The hormone concentrations were prepared as desired. The samples were incubated for 10 min and 200 µl of 6% PCA added. The samples were kept on ice for 10 min and neutralized with 3 M K_2CO_3 to a pH of 6-7. The samples were spun in the centrifuge (MSE) at 2400 rpm (1300g) for

15 min at 4° C. The supernatant was poured off and 3 x 100 µl aliquots of supernatants were placed into each of 3 RT 30 tubes.

The samples were now ready for [^{3}H]-cAMP and followed the standard's procedure as outlined below.

2.13.3 Standard preparation

This experiment was performed on ice at all times. Stock 200 µM cAMP (50 µl) was added to 50 ml of distilled water (if normal standards) or 25 ml of shark ringer and 25 ml of distilled water (if Ringer standards) in RT 30 tubes to give a final concentration of 20 pmol/100 µl of cAMP.

The standards were double diluted using the appropriate buffer from 20 pmol/100 µl of stock to 0.3125 pmol/100 µl of stock and a zero was prepared with 0 pmol/100 µl of stock (either water for normal standards or water/shark Ringer for Ringer standards). Standards were run in both water and Ringer to ensure that the urea in the Ringer did not interfere with the binding of cAMP.

To obtain triplicate standards, 100 µl was added into each of 3 tubes. The same was done for each of the dilutions. To prepare nonspecific binding tubes, 100 µl of stock (200 µM cAMP = 20,000 pmol/100 µl) was added into each of 3 tubes. The three total counts tubes contained 50 µl of [^{3}H]-cAMP (Amersham International plc.,

Buckinghamshire, Eng.) and 450 µl of the appropriate buffer (no charcoal or binding protein was added to total counts). Scintillant was added as indicated below.

To all tubes (except total counts), 50 µl of [^{3}H]-cAMP and 200 µl of diluted binding protein were added and gently vortexed. All tubes were incubated at 0-4^{0}C for 1.5-24 hr.

After incubation, the tubes were transferred to cool centrifuge carriers and 500 µl of charcoal mix was added to each tube (except for total counts) and centrifuged (MSE centrifuge) at 2400 rpm (1300g) for 15 min at 4^{0}C. The supernatant was poured into scintillation vials and 2 ml of scintillant (Ultima Gold XR, LSC-cocktail, Canberra packard, Brook House, 14 Station Road, Pangbourne, Berks) was added to all vials (including total counts) and shaken. All tubes were then counted on the scintillation counter (Packard 1600 TR Liquid Scintillation Analyzer, Canberra Packard, Pangbourne, Berks).

3.0 Results

3.1 Rectal gland histology

Cross-sectional photos were taken at magnifications of x10 (Figures 3.1, 3.2), x25 (Figure 3.3), and x40 (Figure 3.4), illustrating the vast tubule system within the rectal gland. The large central collecting duct (Figure 3.2) can be seen passing through the middle of the gland. The tubules are packed into the gland within the capsule (Figure 3.1), containing nerves, mesothelial cells, and connective tissue. The tubules themselves appear in various shapes and sizes and contain epithelial cells (Valentich and Forrest, Jr. I, 1991; Masini et al., 1994). Connective tissue appears around the periphery of the tubules along with a small capillary network (Figures 3.3, 3.4). The nuclei stained black; muscle cells, tubules, and red blood cells stained red; and connective tissue and mucin stained blue.

3.2 Collagenase exposure time using modified Valentich's method (Method 1)

Boehringer's collagenase exposure times of below 50 min and above 100 min were not able to produce viable epithelial monolayers. Below 50 min, there was a lack of rectal gland tissue digestion. This lack of tissue digestion frequently led to difficulties in attachment of tubules and often led to contamination.

Above 100 min, many single cells were present indicating an

Figure 3.1

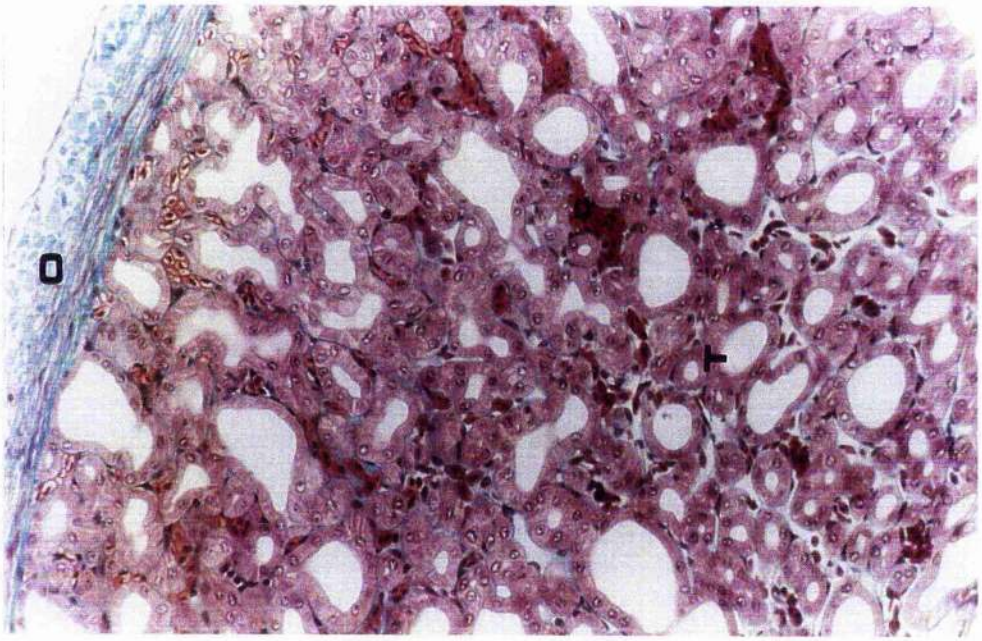
Figure 3.2

Figure 3.1 Cross-section of the dogfish rectal gland (10x) I.

Photo illustrates the branching tubule system (T), the small capillary network (C), and the outer capsule (O).

Figure 3.2 Cross-section of the dogfish rectal gland (10x) II

Photo illustrates the large lumens of some tubules (L) and the central duct (D).


O
L


L
D

Figure 3.3

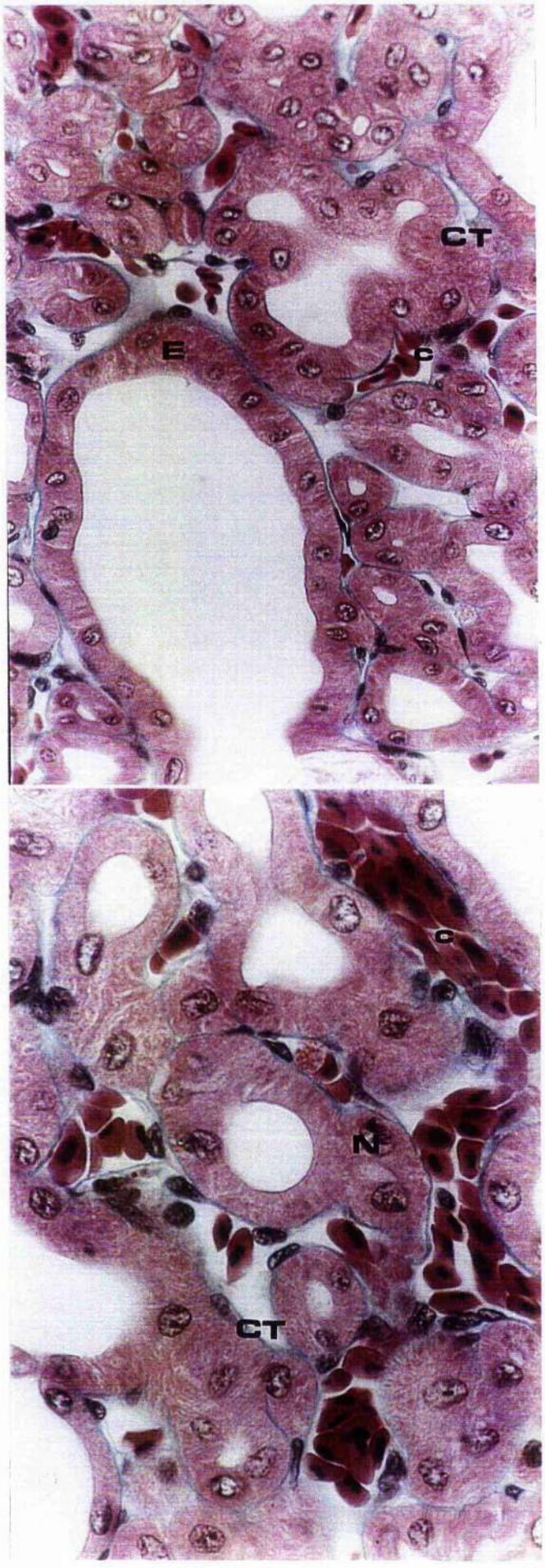
Figure 3.4

Figure 3.3 Cross-section of the dogfish rectal gland (25x).

Photo illustrates the epithelial cells (E) comprising the tubule system, the small capillary network (C), and connective tissue (CT).

Figure 3.4 Cross-section photo of the dogfish rectal gland (40x).

Photo illustrates the nuclei (N) of the epithelial cells comprising the tubule system, connective tissue (CT), and the small capillary network (C).


CT
E
C
C
N
CT

overdigestion. There were few, if any, tubules present. Table 3.1 illustrates the effects of varying collagenase exposure times on the rectal gland. A 90 min exposure to collagenase was found to be the ideal time for optimal cell yield as well as growth and attachment (Figures 3.5-3.9).

3.3 Perfusion technique

Initial attempts at developing a perfusion technique which produced a high yield of tubules proved unsuccessful. Often the problems resulted in overdigestion or underdigestion of the tissue. Overdigestion typically resulted in the production of many single cells which had difficulty attaching to the plate. These single cells were often large and round and although they appeared to be viable with some cells occasionally undergoing cell division, preparations such as these never established an epithelial monolayer possibly due to the inability to attach to the plate.

Underdigestion of the tissue typically resulted in the production of clumps of tissue which also had difficulty attaching to the plate and often resulted in contamination. Occasionally, blood cells would also be found in these preparations.

The final perfusion procedure yielded approximately 400,000 cells/ml with each gland yielding approximately 4-5 ml. Tubules

Table 3.1

Table 3.1 Varying collagenase exposure time using the modified Valentich's technique.

	Time of Exposure to Collagenase-Rocking Time (min)								
Days	20	30	36	45	50	60	90	100	110
1-2	Possibility of tubules; sparcely populated.	Possibility of tubules; sparcely populated.	Clumps of cells present; appears to need more digestion.	Clumps of clearly defined cells and single cells.	Clumps of cells present; appears to need more digestion. Blood present. More dense than 36 min.	Clumps of clearly defined cells and single cells. Many tubules present. Not attaching as well as 90 min.	Tubules not as dense as 60 min, but tubules are attaching and sending out processes.	Tubules not as dense as 90 min. Less clumps. After two days, evidence of epithelial cells.	Not good tubules and appears to be overdigested. After two days, sparce tubules which are breaking up.
3-4	Large, round cells.	Large, round cells.	Some tubules are sticking down and branching out.	Not attaching and eventually lost to contamination.	Tubules are branching out.	Cells are attaching better and sending out processes.	Dense epithelial cells. Better on collagen treated plates.		Lack of attachment.
5-6	Contamin-ation.	Contamin-a tion.					Layer is not complete, but is healthy.		
7-8			More densely populated than 50 min cells.		Big, round cells not attaching, but evidence of epithelial cells.	Epithelial layer not forming. Not a complete layer.	Similar to 60 min in number of epithelial cells at 8 days.	Looks similar to 60 min with respect to epithelial layer.	Few cells in number.
9-10			Epithelial layer larger than 50 min.		Epithelial layer spreading, but not as well as 36 min.	Epithelial layer looks healthy and is expanding, slowly.	Epithelial layer is expanding.	Epithelial layer is expanding and becoming concentrated.	

	Time of Exposure to Collagenase-Rocking Time (min)								
Days	20	30	36	45	50	60	90	100	110
11-12			Epithelial layer becoming more complete.		Poor coverage of epithelial layer.		Epithelial layer is expanding.	Still many big, round cells present; possibly as a result of	
13-14			Still better coverage than 50 min, but not complete.		Some single cells (round) with fair coverage.	The epithelial layer is still not complete, but it is healthy.	Epithelial layer is still expanding and the cells are healthy.	Still healthy, but not a complete monolayer.	Much debris.
15-			Still healthy cells, but not complete layer.			Still healthy, but the layer is not complete.	Best coverage out of all times.		

Figure 3.5

Figure 3.5 11-day old epithelial layer from modified Valentich's method.

The phase photograph shows the presence of mononucleated epithelial cells (M). The fluorescence photograph (below) shows healthy cells each with their own nucleus (green), nucleolus (bright green), and lysosomes (orange-red). (40 mm=100μm)

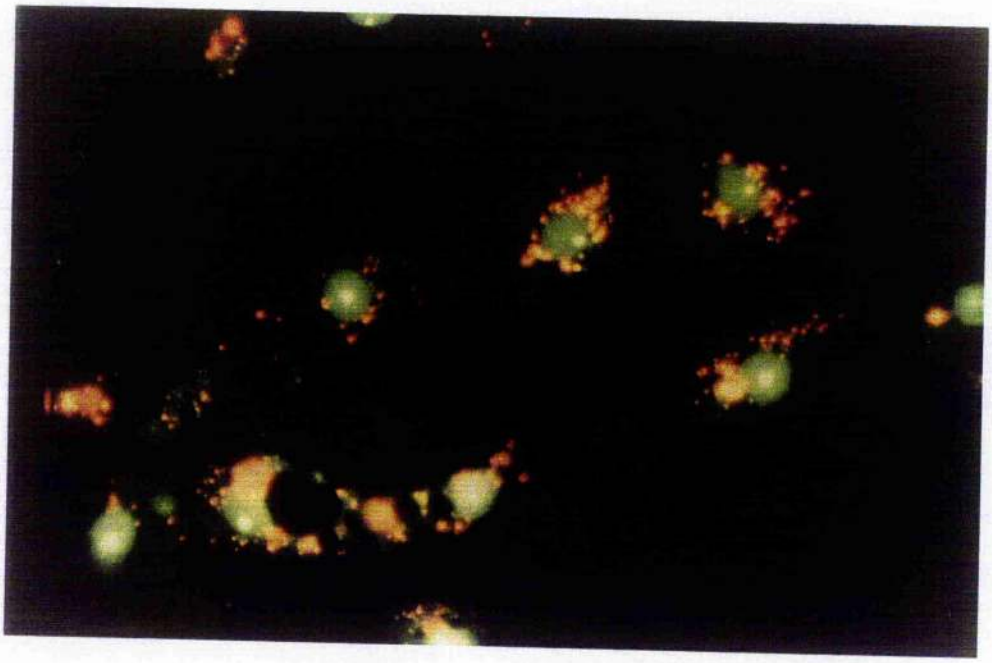
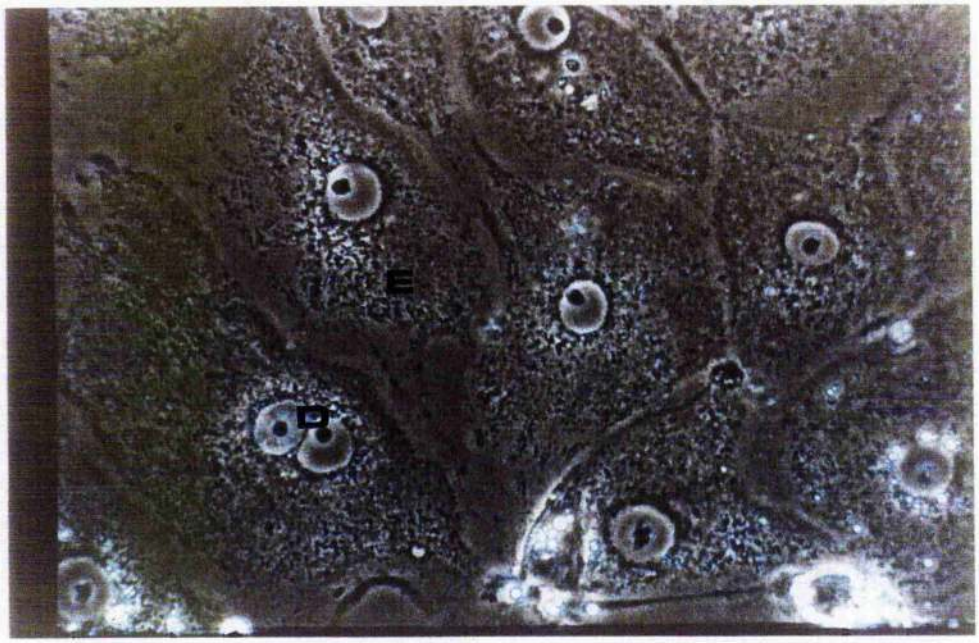


Figure 3.6

Figure 3.6 11-day old modified Valentich's culture magnified 25x.

The phase photograph illustrates healthy granulated epithelial cells (E) and the presence of a dividing nucleus (D). The fluorescence photograph illustrates the healthy nucleus (green), nucleolus (bright green), and lysosomes (orange-red). (40 mm=100μm)


E
B

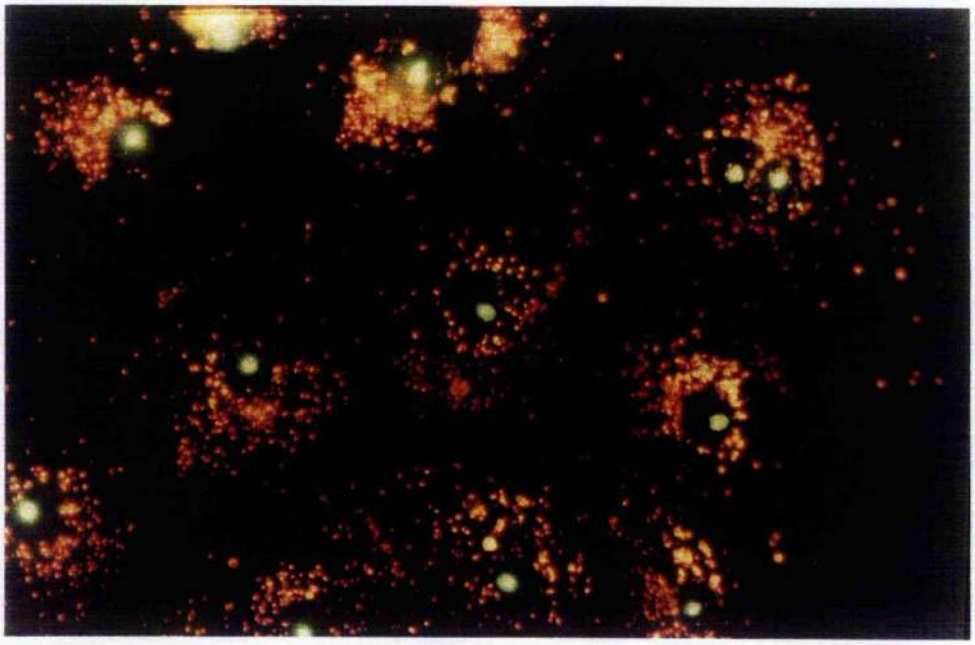


Figure 3.7

Figure 3.7 18-day old modified Valentich's culture.

The photograph illustrates the attachment of a tubule (t) and the resulting outgrowth of cells (64 mm=100μm).

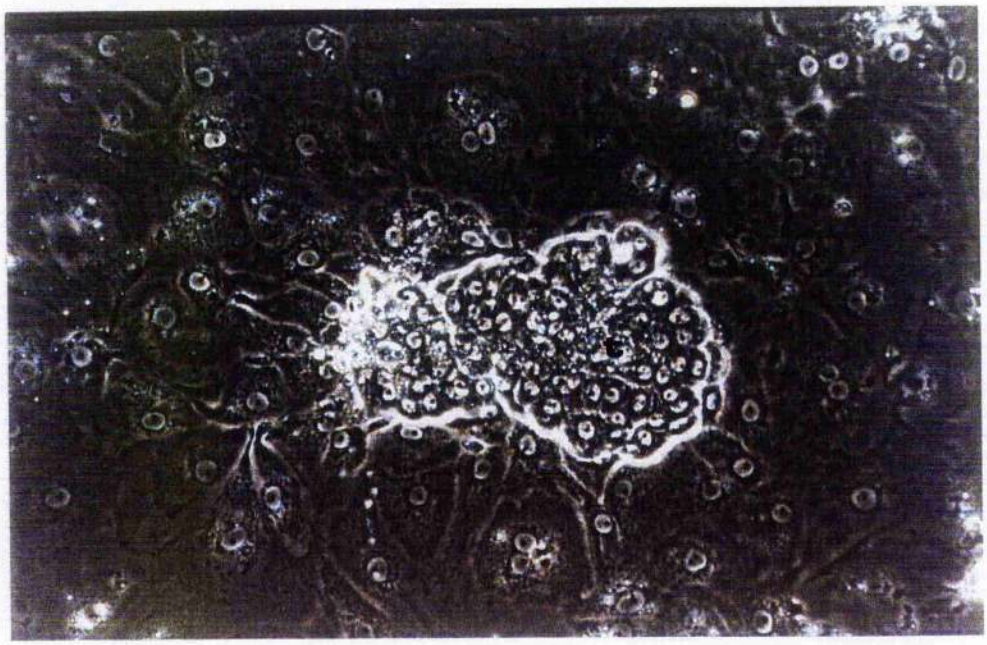
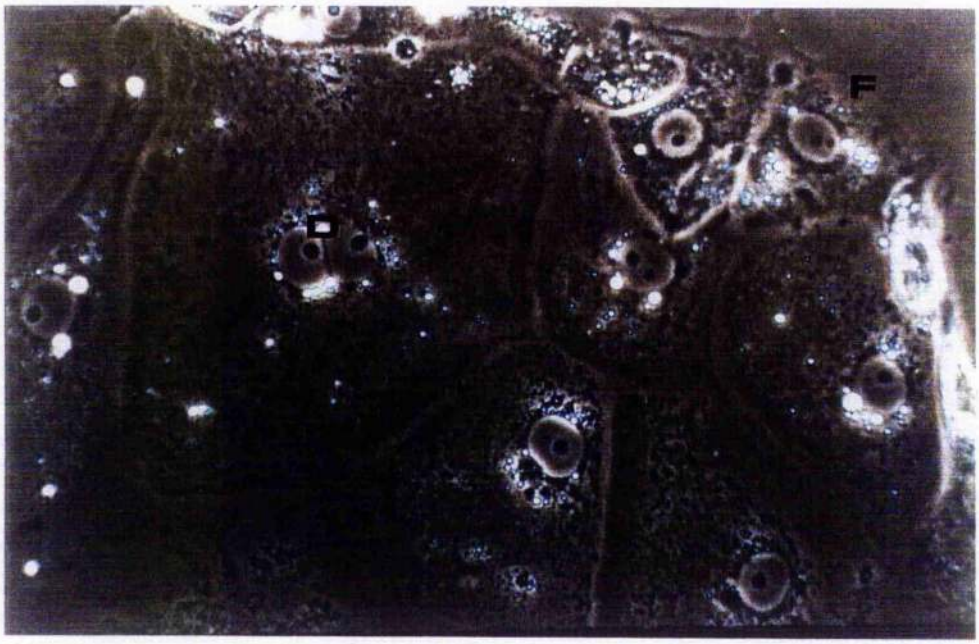


Figure 3.8

Figure 3.8 18-day old modified Valentich's culture magnified 25x.

The phase photograph illustrates the front edge (F) of epithelial outgrowth and two dividing nuclei (D). The fluorescence photograph shows a high density of lysosomes at the leading edge of the epithelial growth (40 mm=100μm).


F
F

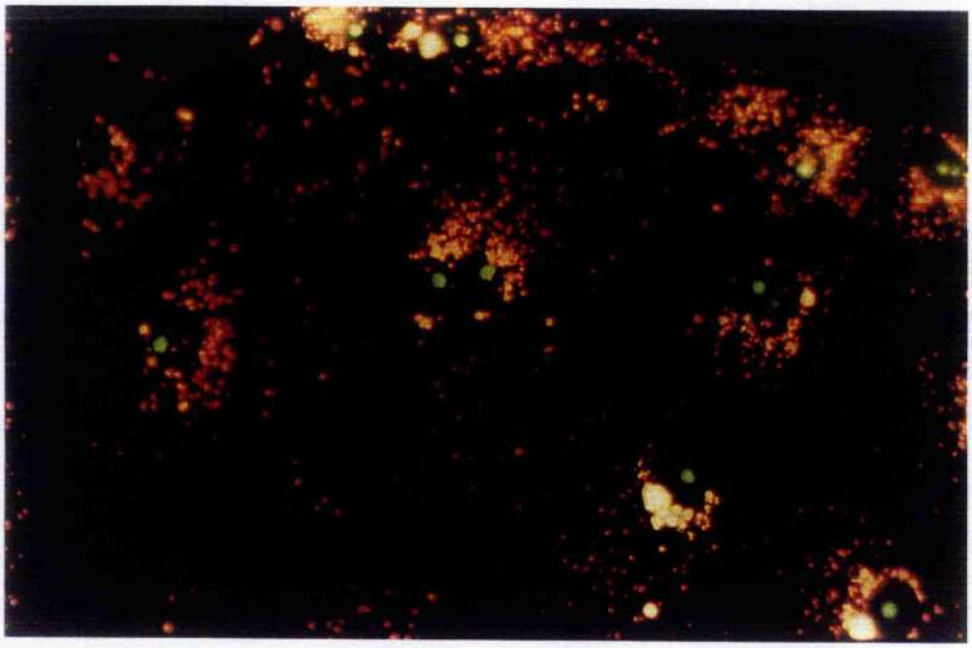
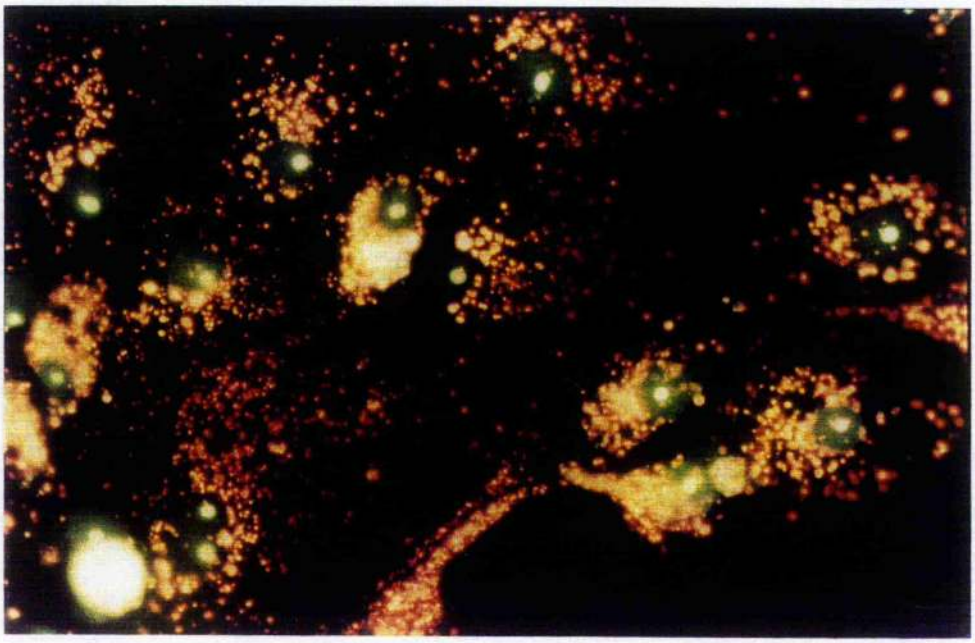


Figure 3.9

Figure 3.9 Flourescence photograph of an 18-day old modified Valentich's method culture.

The photograph illustrates a high density of lysosomes (orange-red), nuclei (green), and nucleoli (bright green) characteristics of a healthy monolayer of epithelial cells (40 mm=100μm).



attached within 2-days with an epithelial monolayer beginning to form after 5-days on both 35-mm plates (Figures 3.10, 3.11) and 96-well plates. After 10-days, a complete monolayer of epithelial cells was seen. There was excellent attachment of tubules. After 17-days, the culture was still healthy (Figures 3.12, 3.13).

Type 1 collagen coating was important to ensure good attachment of tubules. Preparations which were plated onto non-collagen coated plates had difficulty in attaching, leading to poor growth.

3.4 Counting of epithelial cells in 96-well plates

Accurate counts were able to be obtained although counts varied from well to well depending upon the coverage of the monolayer. Counts were normally approximately 22,000 cells per well. The diameter of the cells measured ranged from 11 μm to 28 μm.

3.5 cGMP assay

Exposure to 10^{-6} M sCNP produced a maximal cGMP response at 3 min (from a control value of 3.0 to a value of18.6 pmol cGMP/million cells; average standard deviation of $\pm$0.550) in cultured rectal gland cells (perfusion method) contained in a 96 well plate (Figure 3.14). The assay was performed on a 13-day old culture. The experiment was repeated with similar results were produced.

Figure 3.10

Figure 3.10 Beginnings of growth in a 4-day old perfusion culture.

The phase photograph (top) shows an epithelial cell nucleus (arrow) and the growth front (F). The fluorescence photo illustrates healthy cells (bright yellow; actual color was bright green) with damaged cell fragments (orange-red)(16 mm=100μm).


F

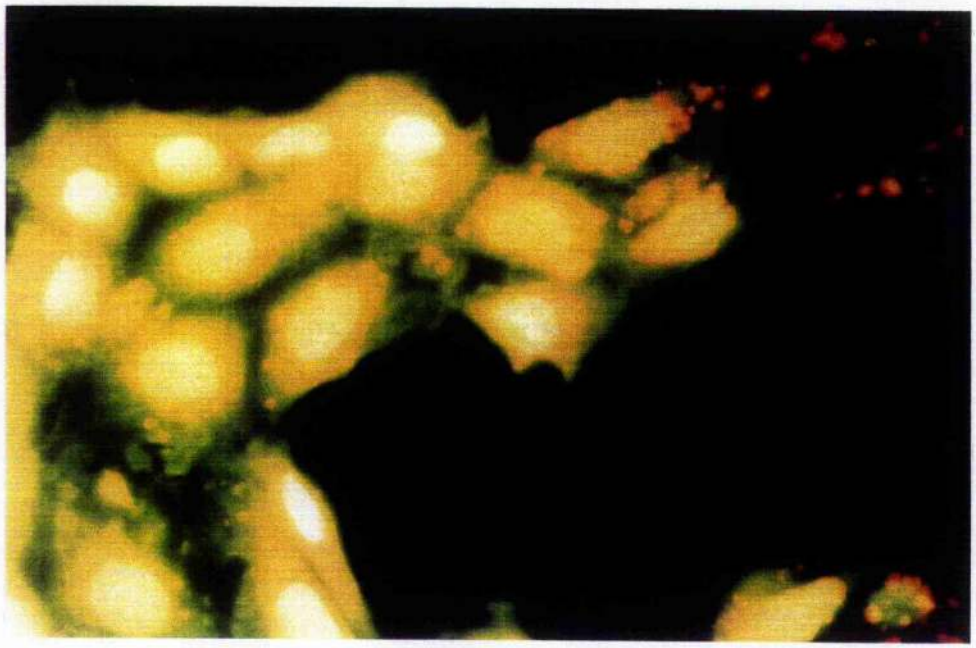


Figure 3.11

Figure 3.11 Beginnings of growth in a 5-day old perfusion culture.

The phase photograph (above) shows the nucleus and nucleolus of an epithelial cell (arrow). The fluorescence photo (below) indicates healthy cells (the cells actually stained more bright green than yellow). The bright yellow circles represent the nuclei (actual color is bright green). Damaged cell fragments stained orange-red (16 mm = 100μm).

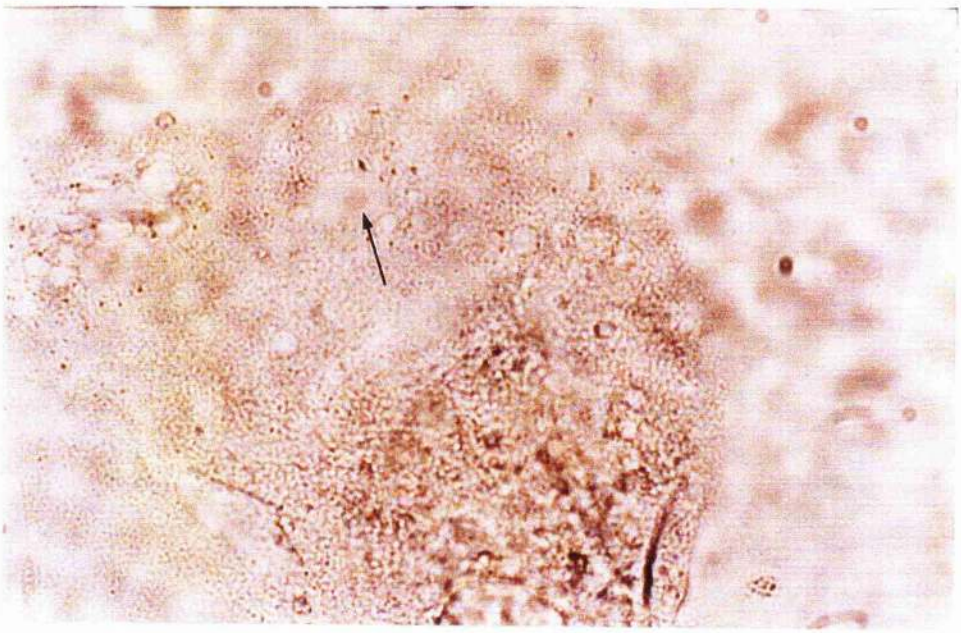
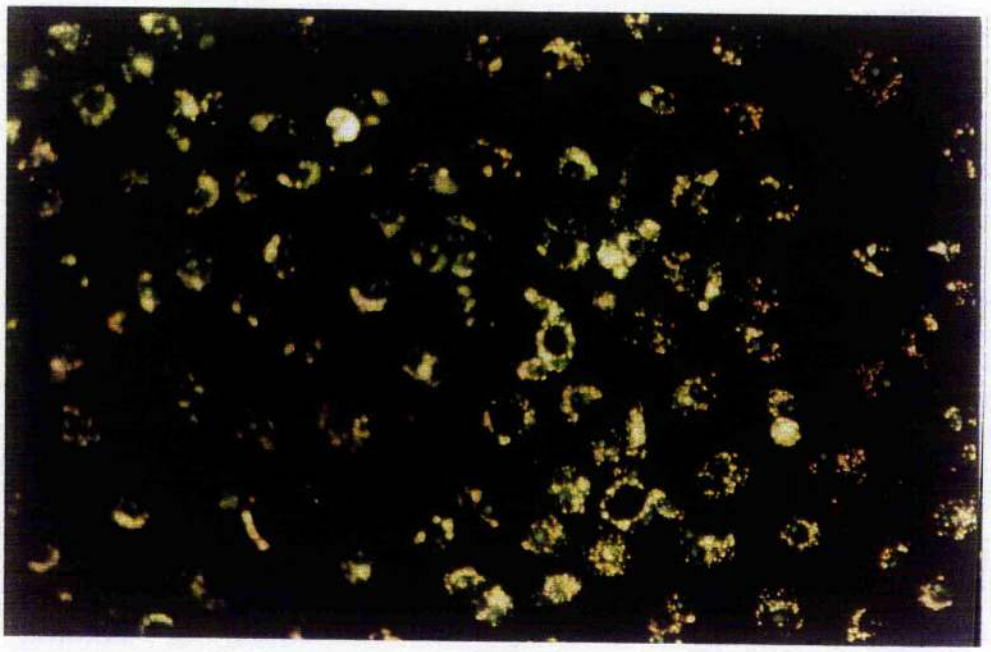


Figure 3.12

Figure 3.12 17-day old epithelial cell monolayer from perfusion technique.

The monolayer shown appears to be healthy from the fluorescence photo (below). The cells are mononucleated (arrow) with the occassional dividing cell. The bright yellow-red in the fluorescence photo indicates the low pH of the lysosomes and the bright green dots indicate the location of the nucleolus (64 mm=100μm).



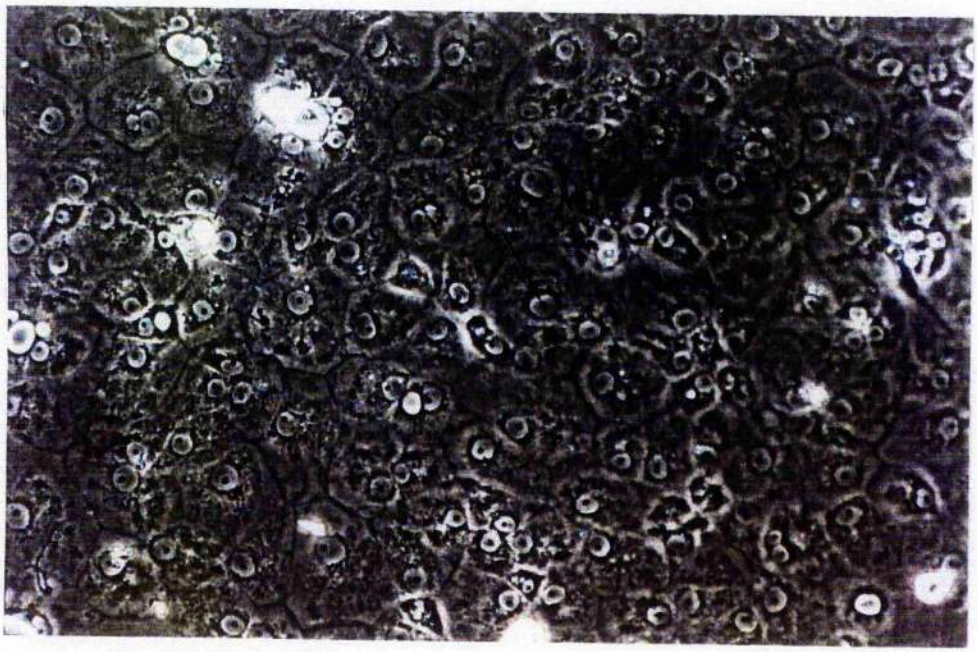


Figure 3.13

Figure 3.13 20-day old culture from perfusion technique.

The photo represents a 20-day old culture with attachment and growth of a tubule (t)(64 mm=100 um).

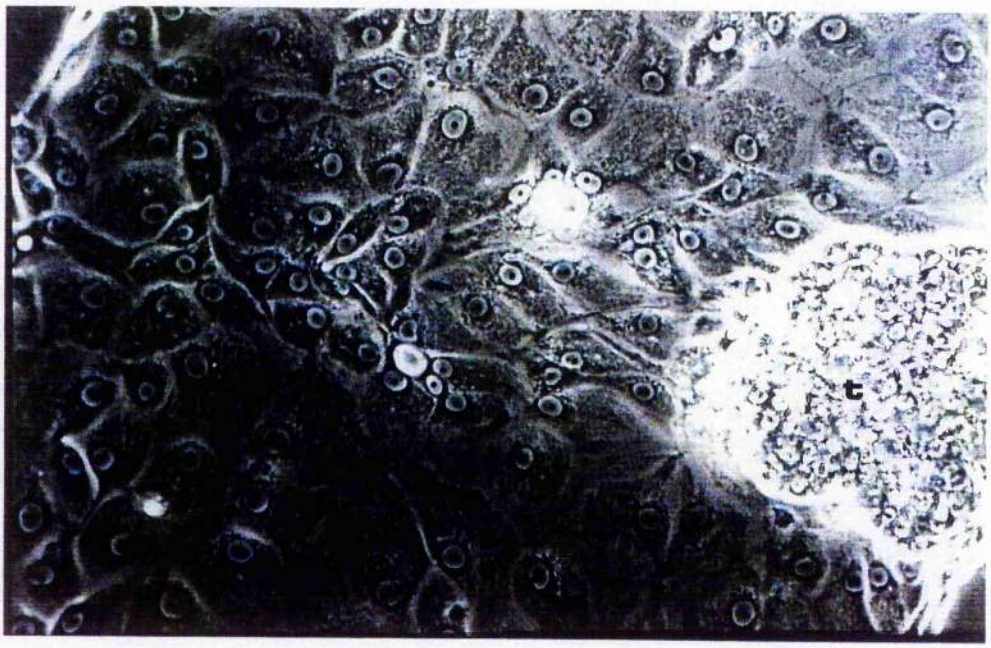
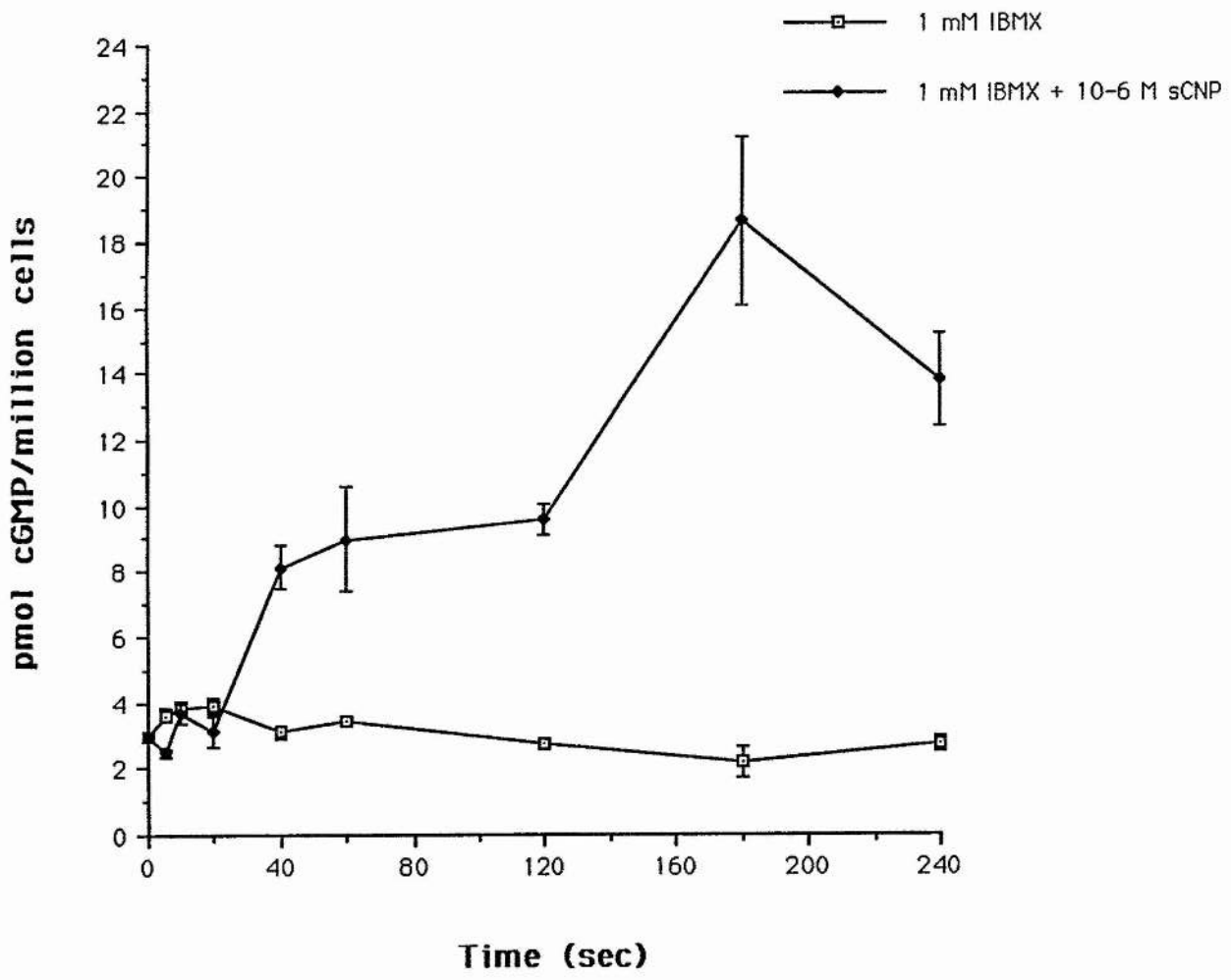


Figure 3.14

Figure 3.14 10^{-6} M sCNP induced stimulation of cGMP production in cultured rectal gland epithelial cells.

Experiment was over a time course of 240 sec.


1 mM IBMX
1 mM IBMX + 10-6 M sCNP
pmol cGMP/million cells
0
2
4
6
8
10
12
14
16
18
20
22
24
0
40
80
120
160
200
240
Time (sec)

3.6 cAMP assay

Accurate results were not able to be obtained using tubules isolated from the modified Valentich's technique due to difficulties in obtaining cell counts and consistent tubule aliquots.

4.0 Discussion

4.0 Discussion

The dogfish rectal gland has become an ideal method of studying epithelial sodium transport due to the abundance of epithelial cells contained within the rectal gland capsule. The epithelial cells comprise a network of tubules responsible for maintaining ion and water balance within the dogfish.

Cross-sectional photos of the rectal gland have revealed these tubules in *Scyliorhinus* stretching from the connective tissue capsule to the interior central canal. Masini et al. (1994) have divided the cross-section into four sections: 1) capsular and subcapsular zones, 2) layer of radial tubules, 3) inner zone of branching tubules, and 4) the central canal. The middle two zones, comprising the main tubule populations, exhibit tubules of various sizes and shapes. These various sizes may be a natural occurrance or possibly enhanced by some outside stimulant. The present study has confirmed this classification of rectal gland structure in *Scyliorhinus canicula.*

Solomon et al. (1984a) noted that rectal gland activity is stimulated due to volume expansion. This increase in vascular volume may be related to an increase in rate of blood flow in the rectal gland. A gland that has undergone a volume expansion may increase the number of capillary beds perfused and therefore, volume of blood flow, to accommodate the extra load. Increasing the blood flow to the rectal

gland, in turn, increases the volume of secretion. Similarly, in a gland from a fish that is not volume expanded, the blood volume may decrease blood flow in capillaries as a means of decreasing secretion.

The rectal gland of *Scyliorhinus canicula* is morphologically similar to the *Squalus* rectal gland except in relation to size. The *Squalus* gland is approximately 5 cm in length compared to the *Scyliorhinus* gland which is about 1-1.5 cm in length.

Initial attempts at culturing the *Scyliorhinus* rectal gland involved the use of perfusion. The gland was perfused via the rectal gland vein instead of the artery due to the fact that the vein was easier to isolate. Retrograde perfusion via the vein did not seem to harm the tissue in any way.

Various perfusion conditions were tested involving calcium- and magnesium-free Ringer soultions with and without EGTA or EDTA. Collagenase of various types from different manufacturers were used at a range of concentrations. Many of the problems encountered in these initial experiments centered on the degree of tissue digestion, leading to poor cell or tubule attachment. If the tissue was under digested, large clumps of tissue, probably composed of under digested cells and connective tissue, typically appeared. These large clumps had difficulty attaching and often became contaminated within a few days. If the tissue was over digested, single cell suspensions resulted which did

appear to be healthy and occasionally under went division, yet were never able to attach to the substrate. A refinement in the technique needed to be developed which allowed for the freeing of the tubules from connective tissue and tight junctions without producing a collection of over digested single cells.

In an attempt to free the tubules from tight junctions which rely on the presence of calcium to function, the following optimal conditions were established. An initial "flush out" perfusion of 5 ml of1 mM EGTA in calcium-free Ringer was used. This first perfusion also accomplished the need to clear the gland of red blood cells. An additional 5 ml of Ringer containing 100 μM $CaCl_2$ and Boehringer Collagenase D (2 mg/ml) was perfused next to digest the tissue followed by 5 ml of calcium-free Ringer. It is important to have the last perfusion calcium-free to prevent the tight junctions from reforming with added calcium.

Once the gland has been perfused, it is often very soft inside compared to the gland before perfusion. This allows the tissue to be carefully scraped free from the connective tissue capsule virtually eliminating the problem of connective tissue contamination while increasing tubule yield.

After scraping the tissue from the capsule, mincing, and suspending the tubules in Ringer, the tubule suspension was passed through two layers of surgical gauze. This proved to be an important

improvement to the technique by removing large pieces of undigested tissue. Culture preparations in which gauze was not used often showed poor attachment and resulted in contamination.

The tubules isolated were viable and attached well to Type 1 collagen coated plates. Attachment proved to be much better on collagen coated plates than on non-collagen coated plates. Due to the size of the glands and therefore the number of tubules, a complete monolayer covering a full 35-mm plate was not often observed, leading to experimentation on 96-well plates. This also proved to be an improvement as cells typically covered a full well. Complete monolayers were achieved typically within 9-10 days with attachment and growth beginning at approximately two days.

This technique could still be improved in the areas of perfusion time and seeding density. Typically, smaller glands seemed to be better digested than larger glands due to the amount of tissue present. Some account needs to be taken to allow for the size of the gland. These changes may come in the form of increased perfusion time gauged to account for the difference in size of the gland. Improvements in seeding density are required because of difficulty with growth in some cultures caused by an over or under abundance of tubules. Cell growth seems to be highly dependent on seeding density. Two rectal glands were often plated onto four 35-mm dishes (1.5 ml/dish; approximately 600,000

cells/dish) or filled fifty-four wells in a 96-well plate (150 µl/well; approximately 60,000 cells/well) at approximately 400,000 cells/ml. These also may need to be varied depending on the size of the gland and the resulting tubule yield. A possibility for measuring tubule yield may be simply weighing the pellet before suspension in culture medium.

In addition to perfusion, Valentich's (1991) method of cell culture was also attempted. This method involves mincing of the rectal gland followed by collagenase digestion. Tubule isolation is carried out followed by three washes in Ringer. Early attempts at this method proved unsuccessful, despite changes in collagenase exposure times, selection of various collagenase subtypes and Ringer volumes, and varying mincing times in order to reduce the appearance of large clumps of tissue or single cells. Total perfusion time ranged from 20 min to 110 min. Due to improvements in the overall technique, 90 min exposure to collagenase was the optimum time with limited success also found at times of 50, 60, and 110 min. Although a complete monolayer covering the entire 35-mm plate was never observed, growth was typically complete within 9-10 days, similar to the perfusion technique.

The size of the *Scyliorhinus* gland posed a few problems using Valentich's technique. Because the gland is smaller than that found in *Squalus*, difficulties arose in the number of tubules which could be

isolated and thus seeding density. One gland was typically not enough to plate one or two 35-mm dishes. Some tubules were occasionally lost in the isolation and washing stages of the procedure, leading to increased difficulty in maintaining optimum seeding densities. In contrast, the mere size of the *Squalus* gland allows for some loss of tubules during the isolation procedure. Therefore, Valentich's technique is more suited for use on the larger*Squalus* gland.

Successful cell counts from the perfusion method were able to be obtained on the 96-well plate. In confluent monolayers of 10-14 days, the number of epithelial cells per well seems to number approximately 22,000. This data illustrates that 96-well plates are able to accommodate enough cells to allow the study of epithelial transport responses to hormones.

Valentich (1991) successfully cultured rectal gland cells in *Squalus acanthias* forming a monolayer of transporting epithelial cells which showed a potent chloride secretory response to 10^{-8} M VIP, 10^{-6} M forskolin, and 10^{-7} M atriopeptin. Killifish CNP also produced a potent stimulatory response at concentrations as low as 10^{-10} M (Karnaky et al., 1992).

cGMP assays were run on tubules isolated in the modified Valentich's technique and on epithelial cell cultures prepared by the

perfusion technique on 96-well plates. The tubules from the modified Valentich's technique presented various problems which prevented accurate results. As mentioned above, accurate cell counts were not able to be obtained, preventing any calculation of cGMP content per cell. It was also difficult, if not impossible, to ensure consistent numbers of tubules in each experiment, therefore making results inconsistent.

cGMP assays tested on cultured epithelial cells in 96-well plates provided an easier and more reliable method of measuring cGMP levels. The increased number of experimental wells provides the opportunity for a wide range of experiments. Typically, 54-60 wells were able to be obtained from two rectal glands. Reliable cell counts were obtained from the wells, allowing for the calculation of cGMP produced per million cells.

cGMP assays were performed on the 96-well plates to test the effects of scyliorhinin II, sCNP, and urotensin II on cGMP production. A hormone has been isolated from the gut of the dogfish (*Scyliorhinus canicula*) named rectin which has been shown to stimulate the secretion rate in the isolated perfused rectal gland of *Scyliorhinus canicula* and produced increases in oxygen consumption in rectal gland slices (Shuttleworth and Thorndyke, 1984). Recently, Anderson (personal communication) has isolated a hormone from the gut of the dogfish (*Scyliorhinus canicula*) through purification of dogfish gut extracts and

structural analysis which stimulates the isolated perfused rectal gland. This hormone, synthetic scyliorhinin II isolated from the gut, produces a stimulation in the rectal gland similar to that produced by rectin and has been shown structurally identical to scyliorhinin II, a previously identified hormone in the tachykinin family (Conlon et al., 1986).

Commercially available synthesized scyliorhinin II has been shown to stimulate a significant rise in chloride clearance rates in the isolated perfused rectal gland of *Scyliorhinus canicula* (Anderson, personal communication). Synthetic scyliorhinin II (10^{-6} M) produced a 2.5-fold increase in chloride clearance rates. Scyliorhinin II (10^{-9} M) produced a 2-fold increase in clearance rates while at concentrations of 10^{-12} M the peptide appeared to have little to no effect. Anderson (personal communication) suggests that scyliorhinin II and rectin may be the same hormone, although a full sequence of rectin has not been obtained to verify this.

Experiments performed on isolated perfused rectal glands test two variables: 1) effects of the hormone on rectal gland vasculature and 2) on the rectal gland epithelial cells directly. Cell culture, on the other hand, eliminates the vasculature variable and focuses directly on the epithelial cells. Therefore, cell culture can be a useful tool in conjunction with isolated perfusion to determine the target site of these

hormones.

Limited experiments with scyliorhinin II yielded inconclusive results in cGMP stimulation with more experiments required to fully test the effects of scyliorhinin II on the rectal gland cultures.

Few experiments were able to be conducted with urotensin II, but preliminary data showed no effects in stimulating cGMP production. Urotensin II was not able to stimulate secretion in the isolated perfused rectal gland (Anderson, personal communication), but did have an effect on blood pressure (Hazon et al., 1993). Further experiments are required to fully test the effects of this hormone on cGMP production.

Experiments done by Karnaky et al. (1992) showed elevated levels of cGMP in response to 10^{-10} M killifish CNP in cultured *Squalus* epithelial cells. Similarly, cultured *Scyliorhinus* epithelial cells exhibited elevated levels of cGMP in response to 10^{-6} M sCNP + 10^{-3} M IBMX. In time course experiments, maximum stimulation of cGMP by 10^{-6} M sCNP + 10^{-3} M IBMX occurred at 3 min (from a control value of 3.0 ± 0.1 to a value of 18.6 ± 2.6 pmol cGMP/million cells). No stimulation was produced by 10^{-3} M IBMX alone. These results also indicate a homology not only between the *Squalus* and the *Scyliorhinus* rectal gland function but also between killifish CNP and sCNP.

Anderson (personal communication) found that clearance rates

were increased by doses of 10^{-8} M and 10^{-7} M sCNP in the isolated perfused rectal gland. Thus, sCNP appears to stimulate directly via the epithelial cells and possibly also via the vasculature within the gland as seen by stimulation of chloride clearance rates in the isolated perfused rectal gland.

Further experimentation needs to be performed to determine a dose response to sCNP and to determine the effects of other stimulants and inhibitors on cGMP production.

Assays were attempted for cAMP production in tubules isolated from the modified Valentich's technique, but accurate results were not able to be obtained for the same reasons listed above in the cGMP assay. Due to time constraints, cAMP assays were not able to be attempted on the cultured epithelial cells. The effects of VIP, forskolin, and urotensin II on cAMP are still to be tested.

The cultured *Scyliorhinus* rectal gland has been proven to be a viable model for testing cGMP and chloride clearance stimulation induced by endogenous hormones. Work is needed to be done to refine the culturing technique to produce consistent cultures and to identify the mechanisms by which the endogenous hormones operate and their degree of stimulation.

5.0 References

5.0 References

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